UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE

Clinical and Experimental Sciences

Design of a Cell-based In Vitro Assay for the Pharmaceutical Potency Testing of Botulinum Neurotoxin Type-A

by

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ABSTRACT

Botulinum Neurotoxin type-A (BoNT-A), from the bacterium *Clostridium Botulinum* is extensively used in the pharmaceutical and medical industries for the treatment of limb dystonia and various spastic movement disorders. Determining the batch potency before release for clinical and therapeutic use is thus fundamental and is currently calculated using the mouse LD50 potency bioassay. Pursuing *in vitro* alternatives is vital for the replacement, refinement and reduction of animals in research (NC3R's), and if an alternative is available, it should prioritise the animal method. Human induced pluripotent stem cells and neuroblastoma cell lines are sensitive to BoNT-A and this project screens cell lines for toxin sensitivity with the intention of selecting the most sensitive candidate for use in a GMP-compliant routine laboratory assay for the testing of potency. BoNT-A proteolytically degrades SNAP-25_{206}, to yield SNAP-25_{197}, thus inhibiting neurotransmitter release and the detection of this cleaved protein determines toxin activity. This project has successfully discovered a cell line for use in an *in vitro assay* demonstrating that the cells are viable for use after 7 days of growth and incubation in toxin for 48-hours provides the most optimum results. Several cell lines were screened, differentiation methods trialled and iCell Neurons, which do not require an extensive or variable differentiation protocol have been selected as the most sensitive. The method in which the SNAP-25 cleavage as a result of toxin activity is detected proved to be challenging, with the three methods originally selected as potentially sensitive rendering inconclusive or unsuitable results. Western Blot was a viable tool in the selection of the cell line, but this variable method is not a suitable GMP-compliant method for the reliable testing of such a potent and dangerous drug product.

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Shellie Frances Davies (Long)

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Definitions and Abbreviations

Abbreviation	Definition
ACh	Acetylcholine
BoNT	Botulinum Neurotoxins
BoNT-A	Botulinum toxin type-A
Botox	OnobotulinumtoxinA Drug Product (commercial product)
Ca^{2+}	Calcium
CD	Cervical Dystonia
ChAT	Choline Acetyltransferase
CNS	Central nervous system
DAS	Digital abduction score assay
DP	Drug Product
ECUVAM	European Centre for the Validation of Alternative Methods
EU	European Union
FDA	US Food and Drug Association
GABA	Gamma aminoisobutyric acid
GABAergic	Gamma aminoisobutyric acidergic neurons
GLP	Good Laboratory Practise
GMP	Good Manufacturing Process
HC	Heavy Chain (of BoNT-A)
HHD	Hailey-Hailey Disease
hIPSCs	Human Induced Pluripotent Stem Cells
HN	Translocation domain of BoNT-A

Abbreviation	Definition
hNSCs	Human Neural Stem Cells
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
ICH	International Conference on Harmonisation
I-type	Intermediate Morphology
KTP	Knowledge Transfer Partnership
LC	Light Chain (of BoNT-A)
LD50	Lethal Dose (50%)
LES	Lower oesophageal sphincter
MCE	Microchip Electrophoresis
MS	Mass Spectrometry
NC3Rs	National Centre for the Replacement, Refinement and Reduction of Animals in Research
NICEATM	National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods
N-Type	Neuroblastic Morphology
NTs	Neurotransmitters
QC	Quality control
RMF	Rat muscle force assay
SNAP-25	Synaptosome-associated protein
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
S-Type	Substrate Adherent Morphology
TH	Tyrosine Hydroxylase
VAMP	Vesicle-associated membrane protein

Chapter 1: Introduction

1.1 Botulism

Botulinum Neurotoxins (BoNTs), defined by seven serotypes A-G are produced by the bacterium *Clostridium Botulinum* and are potent neurotoxins that cause botulism. This rare, but potentially fatal paralysing disease is characterised primarily by flaccid paralysis of cranial motor nerves which causes droopiness of eyelids and peripheral muscle weakness (Shapiro, Hatheway et al. (1998). Subsequent progressive symptoms include blurred vision, dry mouth and limb muscle weakness, which progresses to paralysis and respiratory failure as the diaphragm is affected. This paralytic effect is caused by the presynaptic blockade preventing the release of acetylcholine (Nigam and Nigam 2010). There are three defined routes of botulinum toxin infection allowing toxin, or bacteria spores, to contract into the body. These routes apply to both humans and animals; ingestion of preformed toxin, toxicoinfectious botulism and wound botulism. Ingestion of preformed toxin is commonly associated with foodborne or animal botulism specifically where food sources are contaminated with formed toxin, which directly penetrates the gastrointestinal mucosa and reaches the neuromuscular junction to lead to its paralytic effect (Jean, Fecteau et al. 1995). Wound botulism is a stand-alone type, affecting both animals and humans, where the bacteria contaminates a wound and produces toxin, which is subsequently taken into the body. Toxicoinfectious botulism is commonly associated with infant botulism. This refers to cases of botulism where there is a continuous intestinal production of toxin due to a colonisation of the clostridial bacteria in the large intestine (Cagan, Peker et al. 2010). This form of botulinum toxin entry is unlikely in adults as protective bacteria and inhibiting bile acids in the intestines does not allow clostridial botulinum to germinate and produce toxin.

These three defined routes can be attributed to each of the defined types of botulism; foodborne, wound, infant, animal, iatrogenic and inhalation botulism. These are portrayed in figure 1.1 and table 1.1 below and described in detail in the following sections.

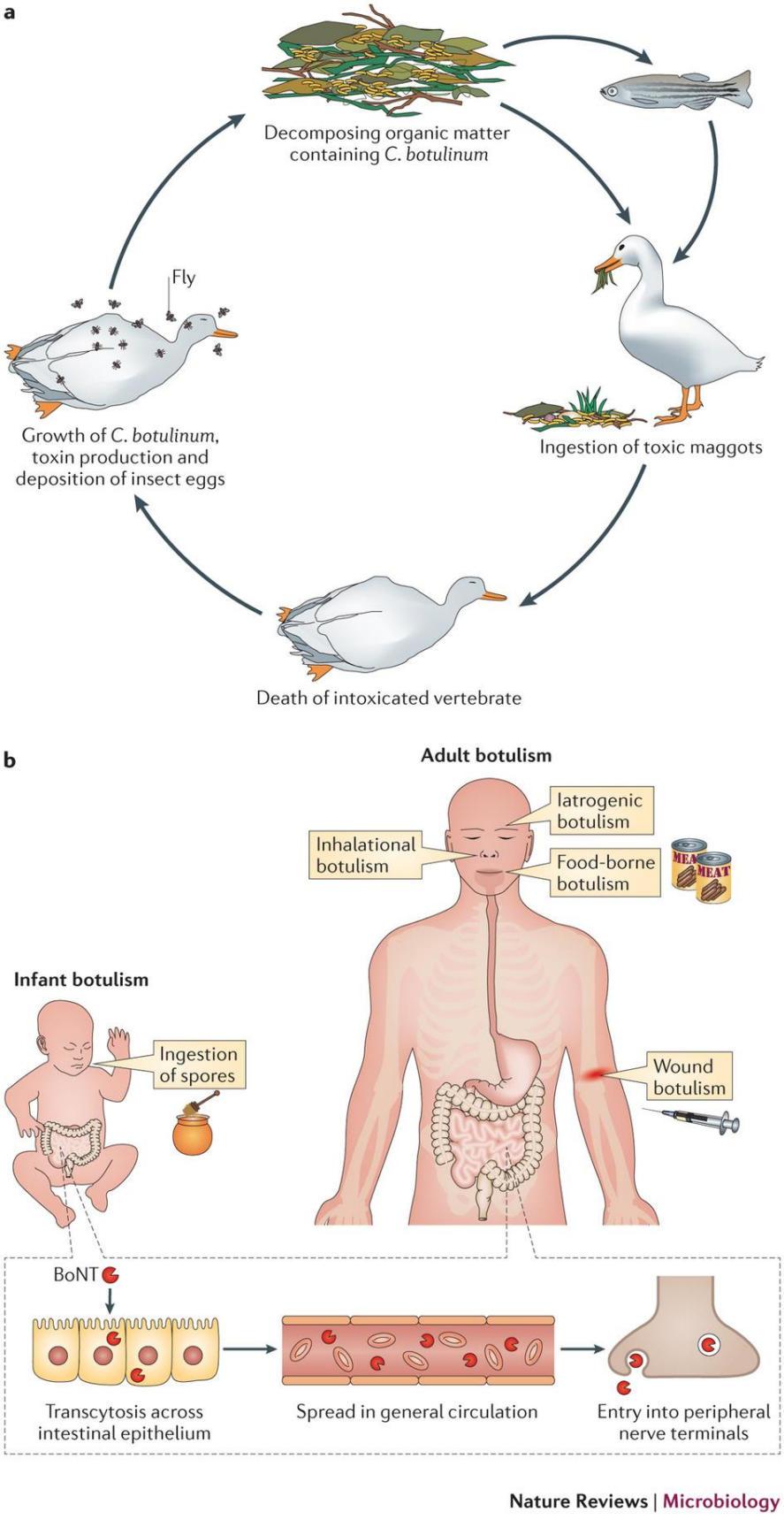


Figure 1.1: Types of Botulism (a) *Cycle of Animal Botulism* ***(b)*** *Types of Human Botulism (Rossetto, Pirazzini et al. 2014). Nature Reviews Microbiology.*

Type of Botulism (route of infection)	Definition
Foodborne Botulism (Ingested toxin)	Toxin is consumed in food, usually in home-canned food (Juliao, Maslanka et al. 2013).
Wound Botulism	The bacteria enters the body via a wound. Cases are also associated with intravenous drug use (Swedberg, Wendel et al. 1987).
Infant Botulism (toxicoinfectious)	Associated with an infection of the intestinal tract of an infant, with descending flaccid paralysis, constipation and lethargy (Morris, Snyder et al. 1983).
Animal Botulism (toxicoinfectious, ingested toxin, wound botulism)	Wild and domesticated animals are infected when contaminated material, such as invertebrates unaffected by toxin, are consumed. This can wipe out large populations very quickly, and can be profuse in areas such as shallow alkaline waters, abundant in invertebrate species and vertebrate cadavers (Rossetto, Pirazzini et al. 2014).
Inhalational Botulism	Botulism occurs following the inhalation of aerosols containing Botulinum toxin. This refers largely to the prospect of BoNTs being using in bioterrorism.
Iatrogenic Botulism	This type of botulism refers to the development of the disease as a result of over-dosing with medicinal or cosmetically used botulinum toxins.

Table 1.1: Types of Botulism

Figure 1.1a demonstrates the cycle of animal botulism, which affects both wild and domesticated animals. Toxigenic clostridia grow in decaying anaerobic material and release toxins. BoNT-insensitive organisms, such as maggots,

consume this decaying material and toxin which are later consumed by other species, thus disseminating the bacterium to vertebrates. When these animals are infected they inevitably die and the cadavers of these intoxicated animals allow the bacteria to amplify and release the toxin (Rossetto, Pirazzini et al. 2014). Figure 1.1b indicates the five forms of human botulism. The most common are foodborne and infant botulism. The three other forms, inhalational, iatrogenic and wound, are much less common. All human and animal affecting epidemiological types of botulism demonstrate the same symptoms, and have the same molecular action which leads to paralytic effect.

1.1.1 Animal Botulism

The rare occurrence of human botulism is contrasting to the reported cases of animal botulism (Critchley 1991). There are well documented cases in horses, cattle, wildfowl and farmed poultry (Jean, Fecteau et al. 1995, Espelund and Klaveness 2014). Animal botulism displays the same symptoms to human botulism; animals are dull, reluctant to move or eat, and flaccid paralysis, typically starting with the hindquarters, is demonstrated, accompanied with muscle weakness and tremors. Abnormal posture of the head can also be seen (Critchley 1991).

Anaerobic environments that facilitate the growth of clostridium botulinum are easily created with the decomposition of plant and animals and this can allow for the entry of the bacteria into the animal food web (Espelund and Klaveness 2014). The cycle of contamination and potential spread of botulism within an animal population is shown in figure 1.1a. The bacteria rapidly create spores, and it has been suggested that in wetlands in particular, botulinum type-C can associate with organisms that are typically unaffected by toxins, which in turn incubates the toxin until consumed by other toxin susceptible organisms. When discussing animal botulism it is typically caused by type-C botulinum neurotoxin, with most cases being reported as type-C and research suggests that farm stock animals, typically cattle, are most sensitive to type-C compared to humans (Jean, Fecteau et al. 1995). As previously mentioned, there are three most frequent routes of botulinum toxin infection; ingestion, toxicoinfectious botulism and wound botulism. All of these routes apply to both animals and

humans, with ingestion being the most common route of infection in animals. This occurs when food containing toxin, 'foodborne botulism' is consumed and the toxin penetrates the gastrointestinal mucosa and reaches the neuromuscular junction via the circulatory system (Jean, Fecteau et al. 1995). Wound infection can result from contamination of wounds where spores germinate within the tissues of the host; this is less documented in the case of animal botulism. Adult toxicoinfectious botulism, commonly seen as symptomatic of infant botulism, is relatively rare among adult animals. An example of this was documented with an outbreak of type-C botulism in cattle that were fed ensiled poultry litter. Poultry litter is commonly used as a feed supplement as a cheap source of protein. Cattle began to demonstrate clinical symptoms and isolation of the infectious bacterium was found in the gastrointestinal contents of the infected animals.

Stagnant water provides optimum anaerobic conditions, with the presence of rotting organic matter and potentially decomposing carcasses. This provides a potent source of intoxication of wild animals (Espelund and Klaveness 2014). Most outbreaks are associated with infected foods and modern farming practices allow for the introduction of botulism into fodder; silage inevitably contains soil traces which could potentially harbour clostridial botulinum spores or toxins.

1.1.2 Foodborne Botulism

Food-borne botulism occurs following the ingestion of the bacterium Clostridium Botulinum, and the subsequent ingestion of the neurotoxin produced by these bacterial spores (McLauchlin, Grant et al. 2006). This epidemiological type of botulism is rare in the United Kingdom, with 62 cases recognised between 1922 to 2005. Cases usually present after patients eat home prepared foods, or inappropriately stored produce but the largest case in the UK was caused by commercially prepared hazelnut yoghurt in 1989 (O'Mahony, Mitchell et al. 1990). Twenty-seven cases of food-borne botulism were reported, with one fatality and clinicians liaised with public health which is a necessary precaution when an outbreak occurs, and it was contained within 4 days. Table 1.2 below shows several other reported cases, and the food source in which the bacterium was able to thrive.

Year	Cases (deaths)	Home Prepared?	Implicated Food	BoNT Type
1922	8 (8)	x	Duck Pate	A
1932	2 (1)	✓	Rabbit Broth	?
1934	1 (0)	✓	Hare	?
1935	5 (4)	✓	Nut Brawn	A
1935	1 (1)	✓	Minced meat pie	B
1949	5 (1)	✓	Macaroni cheese	?
1955	2 (0)	x	Pickled fish	A
1978	4 (2)	X	Canned salmon	E
1987	1 (0)	X	Airline meal	A
1989	27 (1)	X	Hazelnut yoghurt	B
1998	2 (1)	✓	Bottled mushrooms	B
2003	1 (1)	✓	Sausage	B
2004	1 (0)	X	Hummus	A
2005	1 (0)	✓	Preserved pork	B

Table 1.2: Reported Cases of Food Borne Botulism in the UK 1922-2005 (McLauchlin, Grant et al. 2006).

1.1.3 Infant Botulism

Infant botulism is caused by intestinal colonisation with clostridium botulinum bacterium which allows continued intra-intestinal production of toxin (Cagan, Peker et al. 2010). The ingestion of bacteria spores is considered harmless if ingested by an adult as intestinal bile and bacteria can prevent proliferation of spores (Nevas, Lindstrom et al. 2005). However, if ingested by infants less than one year of age botulinum may cause toxigenic disease when the spores germinate and produce toxin in the intestinal lumen. Infant botulism cases have been recognised since 1933, but were often misdiagnosed as hypotonia. The first diagnosed case was reported in 1976 and it is now the most frequent form

of human botulism in the United States (Nevas, Lindstrom et al. 2005) (Pickett, Berg et al. 1976).

Several causes of infant botulism have been identified; most commonly it is associated with the ingestion of honey, there have been some cases of contaminated formula milk powder and also cases related to dust and soil in the home. The spores of BoNT-producing clostridia can be abundant in soil, dust and aquatic sediments and it can be considered that infants are easily exposed to these potential elements (Abdulla, Ayubi et al. 2012). For most cases of infant botulism the causative agent is not identified and it assumed that the bacteria spores are swallowed from the environment (Abdulla, Ayubi et al. 2012). Honey is frequently associated with causing infant botulism as it is a thriving environment for the germination, survival and incubation of clostridium botulinum spores (Arnon, Midura et al. 1978). There have also been reported cases of botulinum type-A having been recovered from dried rice pudding and botulinum type-B from infant formula milk powder (Brett, McLauchlin et al. 2005).

Although approximately 98% of affected infants demonstrate clinical symptoms between one and six months of age, infant botulism can be presented as early at the first week after birth or up to 12 months of age (Cagan, Peker et al. 2010). Isolated cases of botulinum type-E, C and F have been documented but the primarily causative types are both A and B (Armada, Love et al. 2003).

The hypothesised incubation period for the development of infant botulism is suggested to be 3-30 days after exposure or ingestion of spores (Cagan, Peker et al. 2010). Typically, patients displaying symptoms of infant botulism display constipation, followed by extremity weakness and respiratory compromise.

1.1.4 Wound Botulism

Disturbingly, wound botulism is becoming the most abundant in the United Kingdom, in recent years the numbers of cases have increased, with no reported incidents of wound botulism before the year 2000 (Brett, Hallas et al. 2004). Since then, the cases of this type of botulism have increased in frequency, with a high associated with the use of injected or snorted drugs such as heroin and

cocaine. As previously mentioned, all cases of botulism present with the same symptoms (McLauchlin, Grant et al. 2006); cranial nerve neuropathy, dry mouth, slurred speech which progresses to respiratory difficulties and in extreme, or misdiagnosed cases which allowed for progression of the disease, the need for respiratory support with a ventilator. Early cases of wound botulism were reported in the U.S.A in 1951, with patients typically being those whom had used recreational drugs and developed botulism through traumatic wounds. Historically, wound botulism has only been reported to have been caused by type-A and type-B botulinum toxin (Brett, Hallas et al. 2004) (Brett, Hood et al. 2005).

1.1.5 Inhalational Botulism

The concept of botulinum toxin as a potential inhalation poison is relatively new in the field. The toxic product has always been considered a threat due to its high toxicity and potential to be used in acts of bioterrorism, but the possibility that the toxin could be absorbed through the respiratory system is a different, if not more troublesome cause for concern. Whilst there is abundant research on the ingestion of botulinum toxins through the gastrointestinal there is limited indication as to the effects of toxin transmission through the respiratory system. Recent research has provided evidence that botulinum toxin can be used in aerosol form; a potentially devastating issue when it comes to bioterrorism or an indication that development of inhalation vaccines could be developed (Park and Simpson 2003).

BoNT-A can easily penetrate the gut epithelial cells in cases of ingestion botulism so experiments by Park and Simpson (2003) evaluated toxin entry through human and rat alveolar origin epithelial cells to confirm the capability of toxin translocation. They identified that pure BoNT-A crossed polarized monolayers and crossed epithelial membranes and caused toxicity (Park and Simpson 2003).

Inhalation botulism is not a natural form and is typically rare. Accidental human exposure was documented in a German laboratory in 1961, (Dhaked, Singh et al. 2010) but cases are mainly those of intended experimentation; on Rhesus monkeys for example.

1.1.6 Iatrogenic Botulism

Local injection of botulinum toxin type-A is a well-recognised treatment for both clinical conditions and aesthetic use. The therapeutic use of BoNT-A is well-known, safe and effective method used to treat spasticity movement disorders and it is also used in the cosmetic industry for the reduction of facial lines that digress with age. Iatrogenic botulism presents with generalised weakness and respiratory distress but it is typically rare in patients that have botulinum toxin injections, (Coban, Matur et al. 2010) as the drug product has usually undergone extensive potency testing before product release for patient use. This form of botulism is caused inadvertently, and is likely caused by haematological spread of the toxin (Dhaked, Singh et al. 2010) (Coban, Matur et al. 2010). Four cases of iatrogenic botulism were reported in Florida in 2004 following patients receiving cosmetic injections with a toxin drug product that was not approved for human use (Chertow, Tan et al. 2006). A study by Coban (et al, 2010) presented four cases of patients who developed botulism following therapeutic doses of BoNT-A for spasticity conditions and a further two cases were identified with the administration of onobotulinumtoxinA (Botox™) (Ghasemi, Norouzi et al. 2012). These cases are however relatively rare, as the medicinal use for botulinum toxins becomes more abundant in the health care system, and clinician awareness of adverse effects and patient specific optimum dose is becoming more developed. Most patients completely recover, and the symptoms are only temporary, but administration of an antitoxin vaccine may be necessary to prevent complications.

To summarise, botulism is a potentially fatal paralysing disease that if left untreated can result in respiratory failure and death. The most common human forms are foodborne, infant and wound botulism, and animals are primarily affected by foodborne botulism with some cases of wound botulism being reported. Inhalation of neurotoxins is a relatively new exploratory field and focusses on the potential of botulinum toxin use as a bioterrorism agent. Iatrogenic botulism is also rare, and refers only to patients administered botulinum toxin injections for therapeutic or aesthetic use. These two forms of botulism do not occur in nature. Treatment of botulism, for each type, involves

the inactivation of the toxin in the body. Antitoxin is injected intravenously and neutralises all toxin unbound to nerve endings. This means that it is most effective in early diagnosis, to minimise the paralytic effect.

Whether the bacteria or toxin is ingested, develops in a tissue as a result of a wound or a drug product is purposely injected into the muscle, botulinum toxins are transported to the neuromuscular junctions via the circulatory system, targeting the cholinergic synapses that mediate acetylcholine neurotransmitter release. This targeted action of the toxin leads to the recognised symptoms of botulism disease, with the cleavage of SNARE proteins, which in turn leads to muscle paralysis. The following sections of this chapter describe the clinical aspects and advantages of treatment with BoNT-A and the molecular and cellular action of the toxin that leads to its clinical affect.

1.2 Clinical Uses of Botulinum Toxin

1.2.1 Clinical Uses and Conditions Treated

BoNT-A is particularly potent in humans (Arnon, Schechter et al. 2001) yet it is extensively used in the clinical and pharmaceutical industries as it can be used to treat conditions such as limb dystonia and various spastic movement disorders (Hayes and Yiannikas 1996, Sheean 2007). The paralytic effects of BoNT-A on the muscles can be used advantageously to treat neurological conditions that relate to hyperactivity of the muscle (Truong, Stenner et al. 2009) and developing research suggests that treatment of glabellar frown lines with BoNT-A can improve major depression (Wollmer, de Boer et al. 2012). Spasmodic torticollis, or also known as cervical dystonia, is a condition commonly treated with the use of botulinum toxin type A injections and a study in 1992 by Anderson et al. reviewed the efficiency of this treatment. 95% of patients reported considerable improvement and pain reduction with an average of a nine week benefit of treatment (Anderson, Rivest et al. 1992).

1.2.1.1 Treatment of Achalasia

Achalasia is a condition of the oesophagus which is defined by the absence of peristalsis when the lower oesophageal sphincter (LES) does not relax during swallowing. A study by Pasricha (et al, 1995) hypothesised that injecting BoNT-A could be an effective treatment in relieving the symptoms of achalasia as blocking the release of acetylcholine may restore the LES to a normal resting state. It was concluded that 90% of the patients initially improved and remission was demonstrated in some patients six months after treatment (Pasricha, Ravich et al. 1995). Improvement of the patients was characterised by significant improvement in oesophageal function and reduced pressure or tension in the LES.

1.2.1.2 Treatment of Hailey-Hailey Disease (HHD)

HHD is a rare disease characterised by lesions and erosions on the intertriginous regions of the body. These lesions typically occur in the axillary (underarm) or inguinal (groin) areas where the skin can rub together and is susceptible to heat and sweat. Friction, humidity, heat and microbic colonisation all contribute to the progressive symptoms of the condition and the patients are prone to secondary infections as a result (Bessa, Grazziotin et al. 2010). Traditionally, HHD is treated with topic corticosteroids and antibiotics, prescribed treatments that are usually effective but can cause detrimental side effects with long term use.

There has been indication that given BoNT-As ability to block cholinergic stimulus, it can subsequently cause a reduction in production of sweat. As heat and sweat are primary factors that can worsen the symptoms of HHD, BoNT-A was suggested to have potential benefits to patients with this condition. Two patients were treated with BoNT-A injections as part of a study carried out in 2010 by G.R Bessa and colleagues (et al, 2010); one with axilla lesions and the second with groin abrasions. Thirty days after a course of two injections it was reported that the patient with axilla lesions demonstrated complete remission and the patient with a groin lesion showed significant improvement and reduction (Bessa, Grazziotin et al. 2010). This indicates the diverse application for BoNT-A in the treatment of symptomatic conditions.

1.2.1.3 Treatment of Cervical Dystonia

Cervical dystonia (CD) in a disorder in which the patient demonstrates involuntary movement of the agonist and antagonist muscles in the neck. The condition generally presents over several years in late adulthood, with the onset age varying on a patient to patient basis (Brashear 2009). CD often results in permanent disability with the denaturing symptoms of the disease and symptoms were previously treated with oral medication or surgery. Surgery for CD includes peripheral denervation or deep brain stimulation (DBS) (Braun and Richter 2002) but more effective treatment has been found with the use of BoNT-A injections (Simpson, Blitzer et al. 2008). The particular muscles that need to be injected to relieve the effects of CD are patient to patient specific;

practitioners will observe the particular movements and distortion of the neck to determine the most effective area for treatment (Brashear 2009).

1.2.2 Toxin Injection Treatment and Therapeutic Effect

BoNT-A is the most abundant serotype product commercially available for clinical use; Botox™, Dysport™ and Xeomin™. There is one type-B product that can also be clinically used, referred to as Neurobloc™.

The toxin has to be injected directly into the affected muscles and doses are tailored according to individual susceptibility and mode of use. Patients require injections at regular intervals, as the use of toxin injections manages the condition rather than cure it and this regularity will be dependent on the condition, pain and individual tolerance to the medicinal effect. Muscle weakness induced by the injection of type A botulinum toxin usually last for three months, with requirement for further subsequent injections at regular intervals for the best therapeutic effect (Munchau and Bhatia 2000). This response and effect will depend on the dose administered, the condition treated and individual susceptibility to the toxin. It has been indicated in research that some patients develop a tolerance to the injections, with the development of toxin neutralising antibodies by the organism (Munchau and Bhatia 2000). Patients that develop this resistance may benefit from the treatment of injections of other serotypes. Commonly used are types B, C and F and the duration of effect differs with each with botulinum type F lasting for two months in patients with torticollis (Houser, Sheean et al. 1998). Botulinum toxin type B can be used for patients who have built up a resistance to BoNT-A, though type B demonstrates a shorter duration of effect and the resistance to type B will develop rapidly if an antibody induced failure of therapy with the use of type A was demonstrated previously (Dressler and Eleopra 2006).

1.2.3 Possible Side Effects of Toxin Treatment

There can be side effects to the therapeutic use of botulinum toxin, dependant on the muscle injection site and the condition which it is treating; patients treated for cervical dystonia have reported dysphagia after treatment (Anderson, Rivest et al. 1992). The more common general side effects associated with the

use of botulinum toxin type A for therapeutic benefit are nausea, vomiting, and dry mouth and there is always potential for a patient to develop a resistance to a toxin serotype. The toxin diffuses into the muscles and tissue and the effect diminishes with the distance from the injection site but there have been cases of the toxin spreading to other muscles with high volume injections (Munchau and Bhatia 2000). Weakness of distant muscles, or generalised weakness due to toxin spreading throughout the body in the blood is very rare (Bakheit, Ward et al. 1997).

1.2.4 Aesthetic Uses of Toxin Injections

As well as clinical use, BoNT-A has a popular cosmetic use for reducing facial wrinkles and lines developed with age (Cheng 2007). Approved by the FDA in 2002, non-surgical injections temporarily reduce glabellar lines, periorbital lines and nasolabial folds around the mouth, with an effectiveness of 8-12 weeks. The toxin takes 24-72 hours to take effect and peaks at 10 days in vivo (Nigam and Nigam 2010). With reference to the 2014 Cosmetic Plastic Surgery Statistics from the American Society of Plastic Surgeons, BoNT-A injections for cosmetic purposes have increased by 748% between 2002- 2014 and over 6 million individual procedures were recorded, making this procedure in the top five most popular cosmetic procedures for 2014. It is suggested that it the most popular cosmetic procedure worldwide with more than 2.2 million cosmetic procedures recorded in 2003, only one year after it received FDA approval for glabellar lines in 2002 (Pitman, 2005) (Batra, Dover et al. 2005). Typically, it is recommended that BoNT-A is used for cosmetic treatment for patients under 65 years of age, but some studies have indicated effective cosmetic treatment in those older than 65 (Cheng 2007). Contrasting to other cosmetic procedures, the injection of BoNT-A is minimally invasive and has an immediate and noticeable effect with a quick recovery time. The neuromuscular blocking capabilities of BoNT-A provides focal weakening of the injected muscles involved in facial movements that contribute to aged look, such as frowning, thus reducing the appearance of wrinkles (Cheng 2007). The table (1.3) below provides a list of typical areas of the face treated, cosmetically, with the use of BoNT-A injections.

Facial Injections of BoNT-A
Vertical lines between the eyebrows
Lines on the bridge of the nose
Squint lines at the corners of the eyes
Forehead horizontal lines
Periorbital lines and nasolabial folds around the mouth
Platysmal bands around the neck

Table 1.3: Typical Cosmetic Treatment Areas for Botulinum Type-A (Jaspers, Pijpe et al. 2011) (Cheng 2007).

The recommended treatment for glabellar lines is a series of five injections per treatment, at a dose of 20U/mL, according to the location and size of the muscle (Cheng 2007). Side effects tend to be related to the injection technique, with redness of the skin, bruising and swelling in some patient cases. Patients with severe wrinkles which would require a higher dose of BoNT-A to produce effect are more at risk for complications, many similar to those typical to the previously discussed clinical therapeutic treatments (Munchau and Bhatia 2000) such as nausea, focal facial weakness and potential 'drifting' of toxin. Correct dilution and handling of BoNT-A are also essential for reducing the risk of adverse outcomes.

1.3 Structure of Botulinum Toxin Type A

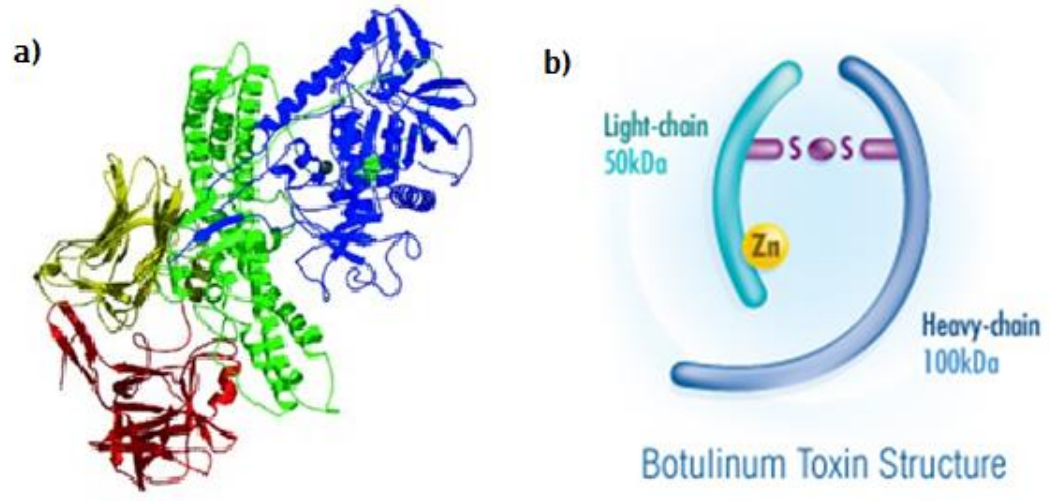


Figure 1.2: Protein Structure of BoNT-A. *Images showing the four domains of BoNT-A and the figure representation of the heavy and light chain.* ***(a)*** ***Blue:*** *Catalytic Domain,* ***Green:*** *Translocation Domain,* ***Yellow:*** *N-terminal Binding Sub-Domain,* ***Red:*** *C-terminal Binding Sub-Domain (Borden-Lacy, et al, 1998, Nature Structural Biology).* ***(b)*** *The light chain and heavy chain linked with a disulphide bond. Image taken from http://jcsciphile.com/molecule-of-the-month/november-botulinum-toxin/.*

The binding domain of the BoNT-A (figure 1.2a), shown in yellow and red, binds to the pre-synaptic nerve ending via gangliosides GD1b or GT1b and the protein receptor SV2 (Dong, Yeh et al. 2006) (Lacy, Tepp et al. 1998). Following cell surface binding and receptor mediated endocytosis an acid-induced change in the translocation domain (figure 1.2a green) allows the passage of the catalytic domain (figure 1.2a blue) across the cell membrane into the cytosol (Lacy, Tepp et al. 1998). The catalytic domain is released into the cytoplasm and is highly specific for the C-terminus of SNAP-25, resulting in the cleavage of 9 amino acids from the protein. All serotypes of BoNT are produced as a polypeptide chain with a molecular mass of 150kD, consisting of a heavy chain (100kD) and a light chain (50kD) linked with a disulfide bond. This is shown diagrammatically in figure 1.2b. The heavy chain (figure 1.2a: shown in red, yellow and green) binds

irreversibly to receptors at the presynaptic surface of neurons and is taken into the cell by endocytosis. The light chain (figure 1.2a: shown in blue) is translocated to cleave proteins from a soluble N-ethylmaleimide-sensitive factor activating protein receptor (SNARE) complex, to inhibit the motoneuron release of acetylcholine, causing muscle paralysis (Dressler and Adib Saberi 2005, Nigam and Nigam 2010). Synaptobrevin, also identified as VAMP (vesicle-associated membrane protein), synaptosome-associated protein (SNAP-25) and Syntaxin have been identified as essential components of membrane fusion function (Blasi, Chapman et al. 1993). Acetylcholine is typically released from the cytosol into the synaptic cleft, but on the addition of toxin, the heavy chain binds to the glycoprotein structures which allows for internalisation of the light chain of toxin. The subsequent result is the inability to release acetylcholine which causes muscle paralysis.

1.4 Molecular and Cellular Action of Botulinum Neurotoxin

1.4.1 Neurotransmitter Release

Signals are passed between neurons and presynaptic cells at the synaptic junction and this signalling triggers the presynaptic neuron to release a neurotransmitter (Lodish, et al, 2007). Neurotransmitters (NTs) are stored in synaptic vesicles and each neuron type typically produces one type of neurotransmitter. The release of neurotransmitters to induce a chemical response is dependent on the ability of the synaptic vesicle to bind to specific receptors in the postsynaptic membrane. The exocytosis of NTs from synaptic vesicles involves vesicle-targeting and fusion to the plasma membrane of the cell. Secretion is permitted following an action potential at the axon terminus being converted into a chemical signal, typically an increase in Ca^{2+} concentration in the cytosol. Ca^{2+} channels are localised to the plasma membrane and membrane depolarisation following action potential opens these channels and Ca^{2+} ions bind to proteins that connect the synaptic vesicle to the plasma membrane (Lodish, et al, 2000). Endocytosis is essential for synaptic transmission and the major endocytic pathway in mammalian cells is mediated by clathrin-coated vesicles (CCVs) (Kozik, Francis et al. 2010) (Lodish, et al, 2007). Internalisation signals are usually in the form of short linear motifs that bind transiently to proteins and protein adaptors. (Kozik, Francis et al. 2010).

The axon terminal has cytoskeletal fibres, which are essential for the localisation of synaptic vesicles to the plasma membrane. Synapsin and a neuron specific GTP binding protein Rab3A are on the membrane surface of the vesicles and are suggested to be involved in the mediation of targeting and fusion at the synapse (Lodish, et al, 2007). Synaptotagmin is also localised to the vesicle membrane and is suggested to be the key protein that triggers vesicle exocytosis. It binds to a complex of plasma membrane proteins, in the SNARE complex which facilitates vesicle docking.

Acetylcholine (Ach) is released by motor neurons at cholinergic synapses with muscle cells, referred to as the neuromuscular junction. This NT is synthesised from a choline acetyltransferase (ChAT) reaction of the acetyl coenzyme A and choline in the cytosol of the presynaptic axon terminal which is then stored in

synaptic vesicles. When released into the synaptic cleft Ach is converted into acetate and choline and enters the plasma membrane. Ach is found in both the peripheral and central nervous systems and is considered the most widely spread and plentiful NT (Waymire, [online] accessed 2017). Ach stimulates muscle contractions and movement and plays a part in the normal function of the cardiovascular, gastrointestinal and urinary tract and in the peripheral nervous system it has been shown to facilitate cardiac, skeletal and smooth muscle contraction (Waymire, [online] accessed 2017).

The proteins that enable the binding of synaptic vesicles to the plasma membrane at the neuromuscular junction are soluble N-ethylmaleimide-sensitive factor activation protein receptors, or SNARE proteins. These proteins are membrane-associated proteins and they facilitate membrane fusion and endocytosis (Duman and Forte 2003). The 25kDa synaptosome-associated protein (SNAP-25) is a presynaptic membrane attachment protein receptor and forms a core complex with SNARE proteins syntaxin and synaptobrevin to mediate synaptic vesicle fusion with the plasma membrane during exocytosis (Hua-Ping Fan 2006). As these SNARE proteins mediate mammalian neuronal exocytosis, the introduction of BoNTs causes flaccid paralysis by inhibiting exocytosis and neurotransmitter release (Chen 2012).

1.4.2 Mechanisms of Toxin Action

Whether botulinum toxin is ingested, absorbed directly from the GI tract or clinically injected into the muscle, it is transported to the neuromuscular junction and targets neuronal synapses.

In a normal neuron, neurotransmitter release is mediated by vesicle exocytosis which is facilitated by the SNARE complex. The synaptic vesicle binds to the synaptic cleft via SNARE proteins and acetylcholine interacts with the muscle cell via acetylcholine receptors; this is demonstrated by figure 1.3a below. When toxin is introduced, the HC of BoNT binds to gangliosides on the presynaptic membrane and receptors on the synaptic vesicle; attempting fusion to the post-synaptic membrane allows the exposure of the protein receptors required for toxin binding. This triggers insertion of the HC domain which facilitates translocation of the light chain (LC) into the cytosol, shown in figure 1.3b.

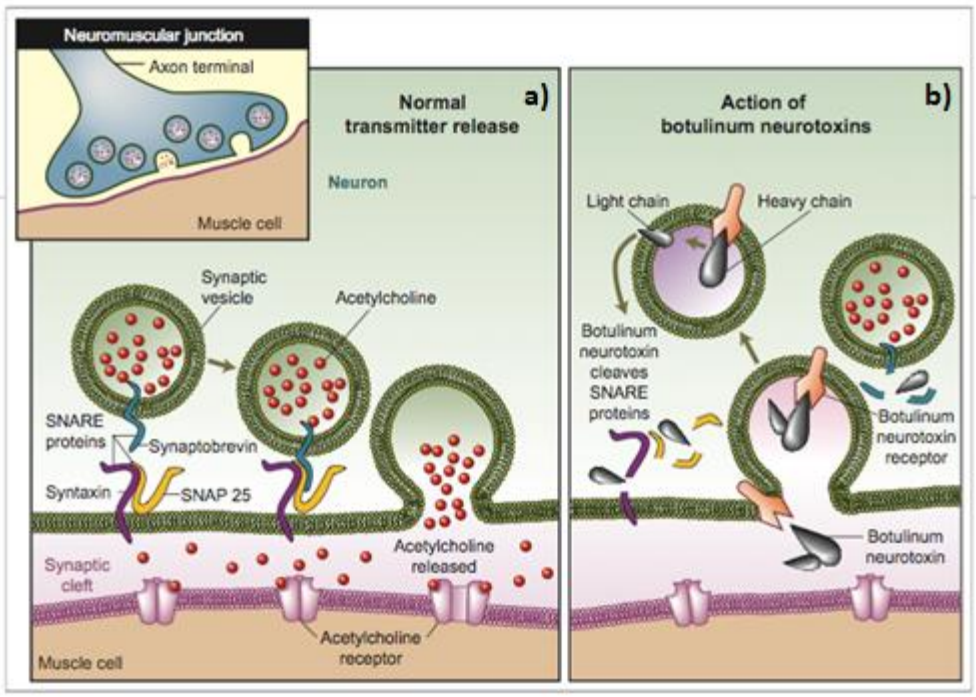


Figure 1.3: Diagram Representation of Toxin Activity at the Synapse. *Process of neuron intoxications by Botulinum Neurotoxin. Figure 1.3 illustrates the normal neuromuscular junction and transmitter release* ***(a)*** *and secondly the activity of botulinum toxin in the synapse* ***(b)****. (Dickerson and Janda 2006).*

When toxin is introduced to the synapse, cleavage of SNARE proteins prevents the binding of synaptic vesicles to the plasma membrane, eliminating the opportunity for the release of the neurotransmitter acetylcholine. The different serotypes of toxin target each of the SNARE proteins specifically; BoNT-B, D, F and G cleave Synaptobrevin, BoNT-C targets syntaxin and serotypes A and E target SNAP-25. BoNT-A, proteolytically degrades SNAP-$25_{1\text{-}206}$, to yield SNAP-$25_{1\text{-}197}$ and SNAP-$25_{198\text{-}206}$ thus inhibiting acetylcholine neurotransmitter release (Christine Saluen 2004).

There are four recognised steps of toxin action in the central nervous system; binding, translocation, internalisation and cleavage of SNARE proteins, resulting in the prevention of acetylcholine release. These steps of toxin activity are discussed in further detail below.

1.4.2.1 Binding of Toxin on the Cell Surface

Botulinum toxin uses the process of synaptic vesicle recycling to enter neurons. The initial step in which BoNTs intoxicate neuronal cells involves the specific binding to the surface gangliosides of the presynaptic membrane through the heavy chain (HC) domain of the toxins; complex gangliosides function as receptors for clostridium neurotoxins (Pickett and Perrow 2011). SV2 on the surface of synaptic vesicles was also proved to be the receptor for BoNT-A, D, E and F, (Dong, Yeh et al. 2006, Fu, Chen et al. 2009, Peng, Tepp et al. 2011), allowing internalisation of the light chain. Synaptotagmin I and synaptotagmin II are the receptors for botulinum serotypes B, C and G (Dong, Richards et al. 2003, Dong, Tepp et al. 2007). Botulinum toxins require complex gangliosides in the neural cell membrane for binding and attachment (Halpern and Neale 1995, Pickett and Perrow 2011). Figure 1.4 below shows the process of toxin binding and its admittance into the cytosol by HC binding to receptors on the vesicle surface and gangliosides on the presynaptic membrane. It has been demonstrated that protein receptors are involved in the binding and internalisation of botulinum toxins, with BoNT-A interacting with the intravesicular domain of the glycoprotein SV2 (Mahrhold, Rummel et al. 2006), a membrane protein localised to endocrine and synaptic secretory vesicles (Sudhof 2004). There are three isoforms of SV2; SV2A, SV2B and SV2C, with suggestion that BoNT-A interacts predominently with SV2C (Dong, Yeh et al. 2006, Mahrhold, Rummel et al. 2006). In the process of presynaptic binding and entry of botulinum toxins into the cell, the toxins bind to polysialogangliosides as well as protein receptors and it has been demonstrated that heavy chain (Hc) fragments of botulinum toxin type A bind primarily to Gt1b (Rummel, Mahrhold et al. 2004). Pre-treatment of cell cultures with gangliosides increases the sensitivity to BoNT-A (Bullens, O'Hanlon et al. 2002). This suggests the concept of the HC domain intially binds to gangliosides to facilitate the anchoring of BoNTs to the plasma membrane surface of neurons. Interactions with protein receptors and gangliosides creates high affinity binding which is critical for the endocytosis process of botulinum neurotoxins.

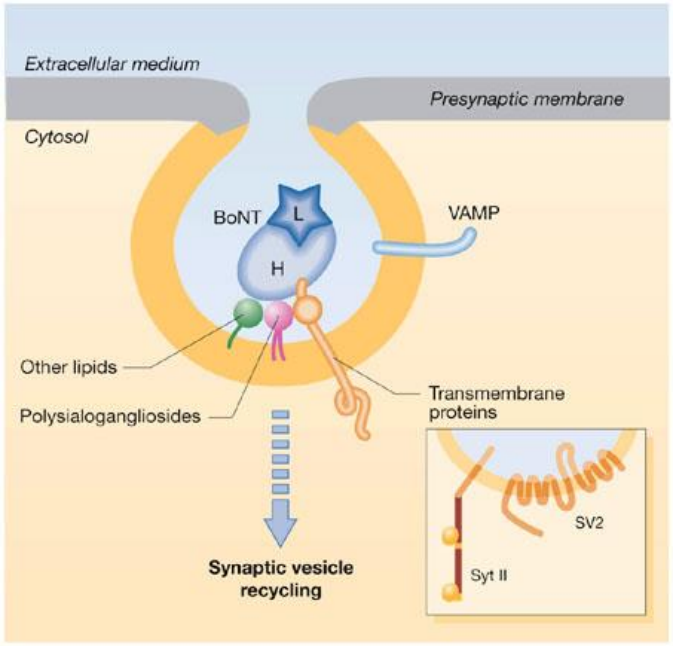


Figure 1.4: Toxin Binding in the Synapse. *This diagram illustrates the role of gangliosides and synaptic vesicle protein in mediating botulinum toxin binding and entry into neurons. (Verderio, Rossetto et al. 2006)*

1.4.2.2 Membrane Translocation of the LC of BoNT-A

The translocation step of toxin activity refers to the movement of the BoNT-A toxin through the membrane, and thus it can be linked to the internalisation step discussed in 1.4.2.3 below. The acidic pH in the lumen of synaptic vesicles onsets a change in the translocation domain of the toxin structure, enabling it to penetrate the lipid bilayer. This subsequently allows the passage of the light chain (LC), attached to the translocation domain, through a formed channel across the membrane (Rossetto, et al, 2014). The disulphide bond crosses the membrane in the later stages of translocation of the toxin and the environmental pH change allows the separation of the LC from the heavy chain (HC) at the translocation domain (Chen and Barbieri 2011). The figure 1.5 below demonstrates this translocation process diagrammatically.

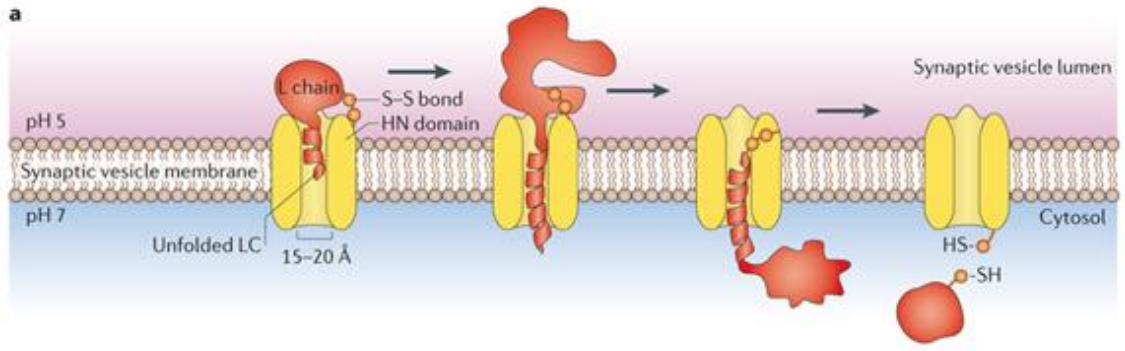


Figure 1.5: Model for Membrane Translocation of the Toxin Light Chain (LC). Image edited from Rossetto (et al, 2014).

As demonstrated by the diagram shown in figure 1.5 the disulphide bond (S-S) crosses the membrane in the later stages of translocation and is reduced on the cytosolic side of the membrane; freeing the light chain from the HN domain. The third step of intoxication of a cell is closely linked to the translocation step and translocation of the LC across the membrane allows internalisation of the LC into the cell itself. Internalisation is discussed in the following section.

1.4.2.3 Internalisation of Type-A Toxin into the Cell

The third step of intoxication of neuronal cells is the internalisation of the light chain (LC) into the cell cytosol. The structure of BoNT-A, described in section 1.3 previously, consists of four functional domains which enable each of the four steps of the toxin activity. The translocation and catalytic domains are involved in the internalisation of the toxin into the cytosol. Following cell surface binding BoNT is internalised through clathrin-dynamin mediated endocytosis of recycling vesicles (Harper, Martin et al. 2011). The translocation domain assists in the internalisation of the LC into the cytosol, as mentioned previously, but the study of this mechanism is limited. It is suggested that the internalisation of the BoNT-A LC into the cytosol is mediated by the charged surface of the translocation domain inserts into lipid bilayers, providing a channel through the membrane allowing the hydrophilic regions of the LC to penetrate and internalise (Chen 2012, Araye, Goudet et al. 2016). The LC passes through the HC channel and

the disulphide bond is broken, suggestively by the neutral pH within the cell cytosol, separating the light chain from the translocation domain. A study by Montal *et al*, describes botulinum toxin HC and LC interaction as a fascinating example of molecular partnership; the HC enables the LC to cross the membrane by instigating an alteration in the pH in the endosome, protecting the LC from the acidic vesicle interior and releases the LC in the neutral cytosolic pH (Montal 2009).

1.4.2.4 Translocation of the LC and Cleavage of SNAP-25

Following translocation, internalisation and detachment from the toxin HC, the BoNT-A LC in the cell cytosol cleaves SNAP-25 which leads to the inhibition of the exocytosis of neurotransmitter vesicles (Chen 2012). A study by Fernandez-Salas (2004) revealed that BoNT-A LC resides in close proximity to SNAP-25_{197} at the plasma membrane, suggesting the reason for its efficient effect on cleavage of SNAP-25, compared to the targeted amino acid region of BoNT-E which resides in the cytosol (SNAP-25_{180}). The duration of effect following treatment with BoNT-A, which is greater than that of type-E (90 days compared to 30 days) is suggested to be a result of partial protection from degradation as it localises to a slow-recycling compartment of the plasma membrane (Fernandez-Salas, Steward et al. 2004). It has been shown that intracellular BoNT-A LC binds directly to SNAP-25, with the N-terminus residues of the toxin LC bound to sequence 80-110 of SNAP-25, enhancing substrate cleavage (Chen and Barbieri 2011). Signal for plasma membrane localisation, LC to SNAP-25, reside within the first eight amino acids at the N-terminus of the light chain, shown by a study by Fernandez-Salas (et al, 2004). They also provided evidence of an active dileucine motif in the C terminus of the LC that is involved in trafficking and interaction with adaptor proteins that form the complexes that mediate the transport of molecules into the cell. It can be hypothesised that the light chain of BoNT-A is directed to the plasma membrane through interaction with these adaptor proteins (Fernandez-Salas, Steward et al. 2004). The active dileucine motif, present at the C terminus of the BoNT-A LC, is unique to type-A toxin and is suggested to play a role in trafficking the LC from the endosomal compartment into the cytosol.

It has been shown that the LC of BoNT-A displayed membrane localisation due to the high affinity binding to SNAP-25, which is also membrane localised. Binding of the LC to SNAP-25 has been shown to occur in two interaction sires; one where the N-terminus of the LC binds to SNAP-25 at the 80-100 region and the second where SNAP-25, at 141-206, binds to the substrate binding cleft of the LC of BoNT-A. Figure 1.6 below diagrammatically shows the high affinity binding of the light chain of botulinum toxin type-A and its interactions with membrane-bound SNAP-25.

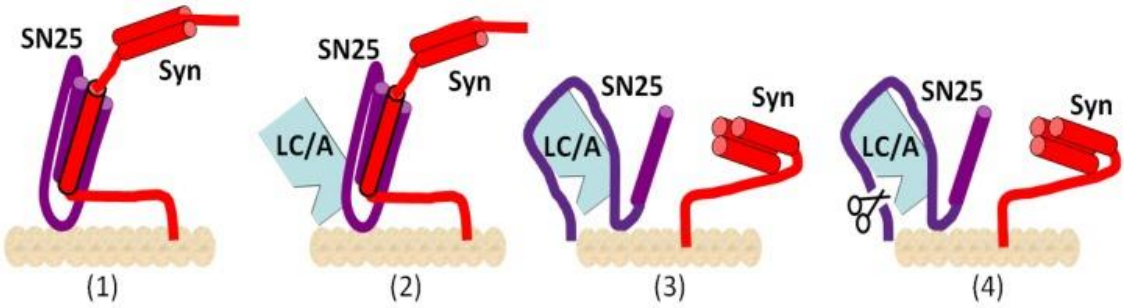


Figure 1.6: Schematic of the Interaction between the LC of BoNT-A and SNAP-25. ***(1)*** *SNAP-25 and syntaxin (syn) form a SNARE complex* ***(2)*** *the N-terminus of the LC binds residues 80-100 of SNAP-25* ***(3)*** *substrate binding separates the core complex* ***(4)*** *SNAP-25 is cleaved at 197 residue. Image taken from (Chen 2012) Clinical uses of Botulinum Neurotoxins.*

The cleavage site selectivity of the LC of all BoNT serotypes is extraordinary; the specificity of each serotype to its targeted SNARE protein is very precise. BoNT-A targets the scissile bond in SNAP-25 (Gln197-Arg198) and BoNT-C targets the neighbouring residue with remarkable distinction (Arg198-Ala199) (Brunger, Jin et al. 2008). The mechanism of the LC recognition and subsequent cleavage of SNAP-25 is complex. Figure 1.7 below shows the crystal structure of BoNT-A LC and the specific binding residues indicated to be involved in the binding of the LC to SNAP-25 that enables cleavage of 9 amino acids from the C-terminus of the protein structure.

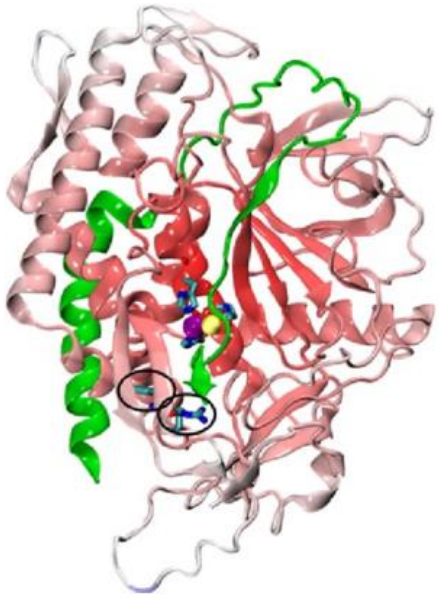


Figure 1.7: Interaction between BoNT-A and SNARE Protein SNAP-25. *Structure of the BoNT-A LC in complex with SNAP-25 shown in green. Image edited and taken from (Pantano and Montecucco 2014). The protease component, shown in red and white, Catalytic Zn2+ shown in purple, associated nucleophile shown in yellow.*

Figure 1.7 above shows the X-ray crystal structure of the light chain of BoNT and its interaction with SNAP-25 following internalisation, taken from (Pantano and Montecucco 2014). The colour in the structure varies from red, which is near the active site, to white suggesting it is further from the active binding site of the toxin LC. SNAP-25 wraps around the circumference of the light chain and the interface between the LC and its substrate is not restricted only to the active site (Brunger, Jin et al. 2008). In the normal functioning SNARE complex, SNAP-25 has a helical formation, shown in figure 1.6(1) above, yet when it is bound to the toxin it can be seen to adopt three distinct forms of secondary structure. The N-terminus residues of SNAP-25 (147-167) form an a-helix, the C-terminus residues (201-204) form a b-strand and residues between are extended. These formations are essential for efficient substrate binding and subsequent cleavage and are termed a-exocites and b-exocites (N-terminus and C-terminus formations respectively). The exocites in close proximity to the scissile bond of the toxin are suggested to ensure the specificity of hydrolysis; the b-exosite on BoNT-A

and key residues surrounding the peptide bond are suggested to be essential in substrate binding (Brunger, Jin et al. 2008). A distinct pocket, referred to as S1 in most studies of BoNT-A structure, near the active site was found to potentially ensure correct specificity to the cleavage site. The exact order in which the LC recognises regions of SNAP-25 is not clear, but several 'couplings' of residues occur to ensure substrate binding. These are shown in table 1.4 below, in a suggested order indicated by research by Chen (*et al, 2011*).

SNAP-25	Toxin Light Chain
P5'-residue Asp[193]	S5'-pocket residue Arg[177]
P4'-residue Lys[201]	S4'-pocket residue Asp[257]
P1'-residue Arg[198]	S1'-pocket residue
P3'-residue Ala[195]	S3'-pocket residue

Table 1.4: Suggested Binding Residues between SNAP-25 and the Light Chain of Botulinum Toxin Type-A (Chen and Barbieri 2011).

It is suggested that the P4' and P5'-residue interactions widen the LC active site cavity allowing the docking of P1'-residue within the S1'-pocket, allowing precise alignment of the scissile bond. The binding of P3 residue confirms alignment of the P1-S1, facilitating substrate cleavage. After cleavage the P4' residue dissociates from the LC which converts the AS smaller conformation which in turn releases the P1'-residue from the active site. This is demonstrated in detail in figure 1.8 below.

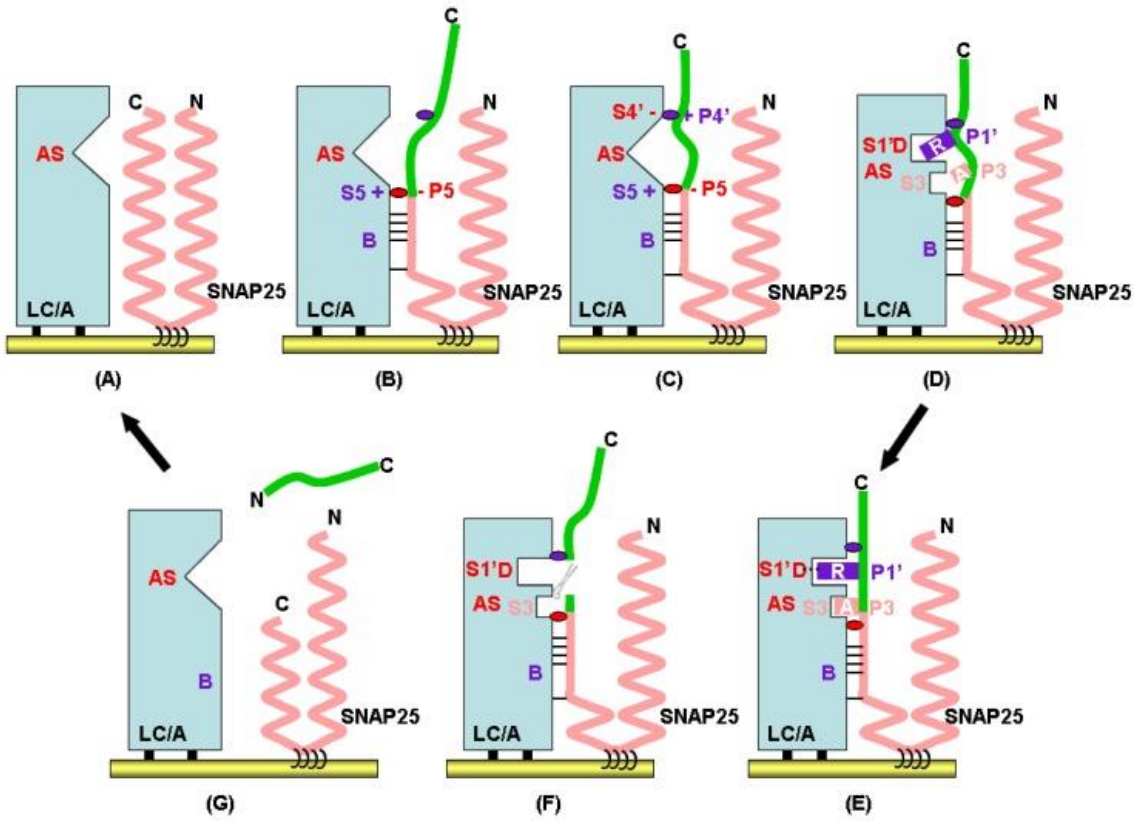


Figure 1.8: Recognition and Cleavage of SNAP-25 by the LC of BoNT-A. (a) *LC binds to the plasma membrane* ***(b)*** *LC binds to SNAP-25, aligning P5'-S5'* ***(c)*** *SNAP-25 is orientated for formation between P4'-S4'* ***(d)*** *docking of P1'-S1' following P3'-S3' binding* ***(e)*** *fine tuning of alignment of the P1' and P3' docking to allow cleavage* ***(f)*** *substrate of SNAP-25 is cleaved* ***(g)*** *the active site returns to original formation. The active site of the LC is labelled as AS, SNAP-25 is shown in pink, with a section shown in green highlighting the area of binding. Image from (Chen and Barbieri 2011).*

Figure 1.8a and 1.8b shows the LC binding to SNAP-25 at the plasma membrane through interactions between residues of the LC and SNAP-25. When bound to SNAP-25 the LC aligns the P5'-residue with the S5'-pocket residue, which is suggested to be the first step in substrate binding according to Chen (2011). This residue binding guides the position of SNAP-25 which thus allows the formation of residue interaction between P4'-residue and P4 pocket, shown in figure 1.8c (Chen and Barbieri 2011). As previously mentioned, and shown in

figure 1.8d, these two steps allow for the broadening and widening of the active site cavity. This enables the docking of the P1'-residue, which is properly aligned with the binding of the P3'-residue to the S3'-pocket residue. SNAP-25 substrate is cleaved and the C-terminal product dissociates from the LC and the active site reduces in conformity (Brunger, Jin et al. 2008).

1.5 Detection of Toxin Activity

1.5.1 Mouse LD50 Potency Bioassay

Batch potency testing of BoNT type-A is fundamental; BoNT is extremely toxic and sample variation leads to the necessity to determine the strength of the product before release. To ensure drug efficacy and the safety of patients, the potency of each batch for release is determined, currently, using the LD50 mouse bioassay. One unit of botulinum toxin corresponds to the median intraperitoneal lethal dose in mice at a defined time point (Sesardic, McLellan et al. 1996). The LD50 can be measured with high precision (Pearce, Borodic et al. 1994) but research indicates that a cell-based assay has the capability of being more sensitive (Pellet 2013). A standard drug product assay uses approximately 250 mice, with the number varying with the assay design; i.e. validation stage, new product assay optimisation. The toxin is injected intraperitoneally to each mouse, and after injection, the mice are observed hourly. There are several defining symptoms that indicate toxin exposure in mice; piloerection, wasp-wasting and shallow breathing. Behavioural alterations are also evident, isolation being a clear indicator. Without humane endpoint intervention, the cause of death would be respiratory failure. Staff are trained to ensure this stage is not met, observing the symptoms described above. The process is time consuming and requires staff observations every hour for the 72-hour test; developing a less time-consuming cell-based method is strongly encouraged in the industry and in compliance with the NC3Rs; reduction, refinement and replacement of animals in research.

BoNT drug products (DPs) are manufactured for pharmaceutical and clinical purposes, and the biological activity of this toxin product needs to be determined to provide drug product safety and efficiency (Adler, Bicker et al. 2010). Ensuring that the correct potency is provided on the release of the DP into the market for use in routine practise is of upmost importance. The current European Pharmacopeia requirements for potency determination are common practise across the EU and ensures that every lot of BoNT product is tested with the mouse LD50 potency assay. The assay is only valid if the dilution range encompasses the LD50 value; at the highest dose 90% of animals are killed and at the lowest, 90% of the mice should survive until the end of the test.

1.5.2 Alternative Methods to the Mouse LD50 Potency Bioassay

Several alternative methods have been researched in recent years, for the refinement, reduction and in some cases potential replacement of animal use in testing. The mouse LD50 has always been considered the optimal or 'gold standard' method but ongoing development of *in vitro* alternative assays in the botulinum toxin industry is important and could possibly provide opportunity to largely replace the mouse potency assay. Some examples of these alternatives are outlined below.

1.5.2.1 Local Paralysis Assays

A less severe test using non-lethal endpoints was promoted as an alternative to the mouse LD50; reducing the severity of the testing and the number of animals used. Several tests are available, but can be difficult to justify when applying for licencing from the Home Office. The comparable sensitivity of the paralysis assays available on the market to the 'gold-standard' mouse LD50 assay is less than desirable, and alternatives for potency testing of BoNTs are encouraged to be complete replacements as opposed to slightly less severe animal testing. The mouse flaccid paralysis assay, the rat muscle force assay (RMF) and the digital abduction score assay (DAS) are the alternative methods available, and with respect to the 3Rs these assays are approved alternatives to the high numbers of mice used in the LD50 assay. Sub-lethal doses are injected in these paralysis assays and the animals generally recover, even at the highest dose (Adler, Bicker et al. 2010). The flaccid paralysis assay involves subcutaneously injecting BoNTs into the inguinocrural region of the abdomen of a mouse and trained technicians score the animals according to the size of the bulge at 24 and 48-hours after injection. The five-point score system is regulated, to prevent differing observations and outlined in table 1.5 below, but it is still commonly thought that the method is too subjective and not comparable in accuracy to the mouse LD50 potency assay.

Stage	Description
0	No signs, normal
1	Just detectable bulge; e.g. covering an area of approximately 0.5cm in diameter or less
2	More pronounced bulge, e.g. covering an area greater than 0,5cm in diameter, but less than the maximum radius of the hind leg heel
3	More extensive bulge extending over a larger area. Extending below hips and top of thigh when viewed from the side and beyond the maximum radius
4	Maximal local effect. More extensive bulge extending over a larger area. Will often extend as far as the bottom of the rib cage, or over a large area with extensive distention or bulging.

Table 1.5: Scoring System for the Mouse Flaccid Paralysis Assay. *Provided from* (Adler, Bicker et al. 2010)

As table 1.5 above indicated, this method of quantification can be regarded as subjective, and therefore not as specific as the mouse LD50 which provides reliable statistical results based on the median dose level.

1.5.2.2 Detection of SNAP-25 Cleavage

SNAP-25 has been identified as having two isoforms; SNAP-25A and SNAP-25B with the B isoform as the most abundant in the adult brain (Bark and Wilson 1994, Yamamori, Itakura et al. 2011). The peptide sequence in figure 1.9 below is representative of the SNAP-25B isoform. A study by Brinkmalm (et al, 2014) identified that the B isoform peptides were in much higher abundance than those of SNAP-25A, with each of their samples containing SNAP-25B compared to a select few containing peptides from the SNAP-25A isoform.

SNAP-25 is 206 amino acids in length and when exposed to BoNT-A, 9 amino acids are cleaved from the C-terminus of the protein (figure 1.9). The long chain of 197 amino acids is the peptide detectable using antibodies for the Western Blot procedure, appearing as a 24kDa band compared to the 25kDa band for the

full-length SNAP-25. The identification of SNAP-25 as the protein specifically cleaved by BoNT-A has enabled the development of several endopeptidase assays, using the detection of SNAP-25 cleavage as indication of toxin activity (Ekong, Feavers et al. 1997). Although these endopeptidase assays have been developed, specific to toxin serotypes, there is high variability, low sensitivity and the experiment is subject to unwanted interference from substrates such as albumin (Jones, Ochiai et al. 2008). The assays detect a SNAP-25 substrate fragment by addition of antibody enzyme reagent which recognises the N-terminus of the cleaved product (Hallis, James et al. 1996) and these results are subject to variation and unspecific results. The assays have been adapted to incorporate antibodies that target various sections of the amino acid sequence of the cleaved product but the assays do not encompass the four steps of toxin activity in vitro (Jones, Ochiai et al. 2008). The need for a robust and reliable replacement for the mouse LD50 bioassay is not fulfilled with the use of these endopeptidase assays.

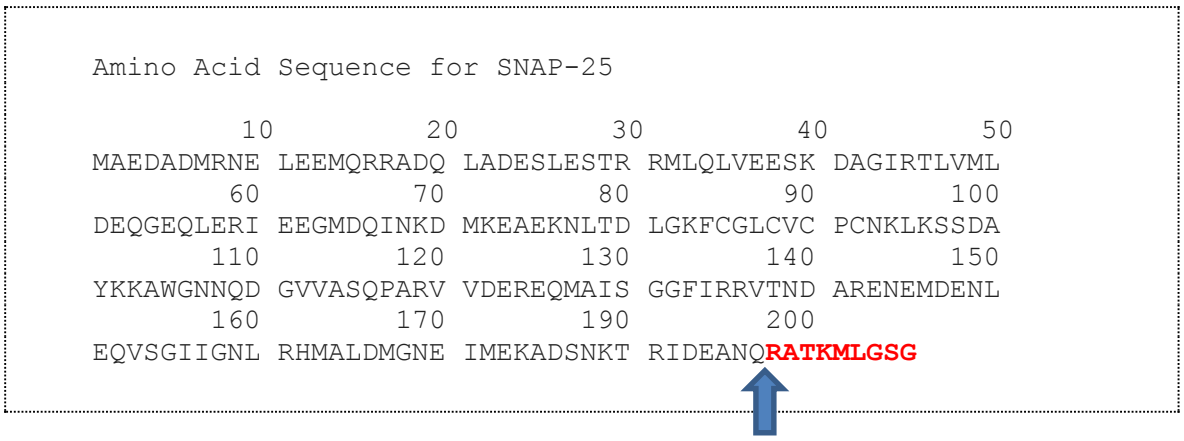


Figure 1.9: Amino Acid Sequence for Human SNAP-25B*. This figure indicates the amino acid sequence for the SNAP-25B protein isoform, highlighted is the 9-amino acid sequence that is cleaved as a result of toxin activity. The cleavage site is indicated with an arrow.*

1.5.3 Progression with the *In Vitro* Testing of Botulinum Neurotoxins

Reducing the number of animals used in research is fundamental, with regard to animal welfare and also from a business perspective with relation to economic benefit; the mouse assay is time-consuming and costly. The National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3R's) is an organisation working with scientists and commercial organisations to discover and apply new technologies to adhere to the 3R's. The necessity to replace the use of animals when alternative *in vitro* methods could be available is pressing, and cell-based assays have been researched in recent years; aiming to exceed the sensitivity of the mouse LD50 bioassay (Pellett, Tepp et al. 2010, Pellett, Du et al. 2011). There have been several publications to suggest that neurons are the most sensitive to BoNT-A, with embryonic stem cell-derived motor neurons, neuroblastoma cells and glutamatergic neurons identified as particuarly sensitive (Kiris, Nuss et al. 2011, Hubbard, Gut et al. 2012, Whitemarsh, Strathman et al. 2012). In particular, murine embryonic R1 stem cell-derived neurons (McNutt, Celver et al. 2011), SiMa neuroblastoma and Neuro- 2a cell lines (Fernandez-Salas, Wang et al. 2012) have been published to demonstrate sensitivity to BoNT-A.

Along with the toxin sensitivity of the cells used in the assay, the method of detection of SNAP-25 cleavage is a limiting factor of the final assay sensitivity. The final assay must accurately quantify small amounts of toxin, be easy to use, robust and reproducable and transferrable to a laboratory setting. Western Blot has been used in published research as the detection method, but quantifying bands based on scanned film images was deemed unsuitable for a GMP method for potency testing; the method has too many variables which could reduce the robustness and repeatability. To find a novel detection method would provide a unique opportunity in the business market; potentially reducing the cost, increasing the number of assays possible with smaller samples, for example.

1.5.3.1 Requirements for the *In Vitro* Cell-Based Assay

It is a FDA (US Food and Drug Association) recommendation that a cell-based assay for product potency testing should incorporate the identified four steps of botulinum activity *in vivo* (Verderio, Coco et al. 1999, Dickerson and Janda 2006) recapitulating the toxins mode of action. These steps were described in detail earlier in this section but to summarise, the steps are as follows;

- Binding: The heavy chain of the toxin binds to glycoprotein receptors on the cell surface.
- Internalisation; the toxin is internalised by receptor mediated endocytosis
- Translocation; Botulinum neurotoxins undergo a pH dependent structural rearrangement in the cell which enables penetration of the lipid bilayer, translocating the light chain of the toxin into the cytosol.
- Cleavage of SNARE proteins; the cleavage of these proteins inhibits the release of acetylcholine, leading to inhibited neurotransmission.

The recapitulation of the four steps is a prominent recommendation from the FDA for the cell-based assay to be approved. It must show repeatability and stability, as well as be a more sensitive detection model in comparison to the mouse bioassay.; approval will not be granted for a replacement method unless better than the original and currently used mouse LD50 method. Deviation from incorporating the four toxin activity steps will provide a less sensitive method than the mouse LD50 bioassay.

Chapter 2: Project Plan

2.1 Industry Project

This project is funded as a Knowledge Transfer Partnership (KTP) by Wickham Laboratories Ltd, a pharmaceutical company that provide potency testing of Botulinum Toxin for several clients, and by Innovate UK. The KTP project is funded for three years, and the programme is designed to help businesses to improve their competitiveness and productivity through the use of knowledge, technology and skills that reside in a knowledge base (University of Southampton). Establishing a collaboration between industry and academia provides an opportunity to utilise knowledge from experts in the field to yield both technical and economic benefit in a new area of research in a business or company.

Commercial pressures, such as competitor's development of a cell-based assay and funding restrictions, has ensured that this project has a strict timeline (see Appendix A- Work Plan). A regular client of Wickham Laboratories supports the associate and company in the project, and two commercial companies thus dictate the requirements and timelines of proof of concept for a sensitive cell-based assay method.

2.2 Project Aim

The overall project aim is to design a novel cell-based assay for detection and quantification of Botulinum toxin type-A activity and potency, applying the principles of the 3Rs concept in the refinement, reduction and replacement of animal use in the potency testing of pharmaceutical botulinum toxin.

For a cell line to be defined as 'sensitive' to toxin, the cells should express robust and repeatable quantities of SNAP-25 protein to enable the quantification and detection of toxin activity via SNAP-25 cleavage. This cleavage is required to be subsequently detectable using the selected detection method to design a complete assay.

Selecting a cell line that is sensitive to toxin, and a detection method that is sensitive enough to detect toxin activity using this cell line are the two defining and limiting factors in the approach to designing a sensitive and specific cell-based assay for the pharmaceutical testing of Botulinum neurotoxin type A.

Therefore, the project has two aims;

1. Selection of a cell line sensitive to Botulinum toxin type-A.
2. Selection and optimization of a detection method for quantifying Botulinum toxin type-A activity and determining its potency.

2.3 Selecting and Characterising a Cell Line Sensitive to Botulinum Toxin Type-A

Human cell lines were selected for screening based on previously published botulinum toxin sensitivity results (Pellet 2013). Researchers have published data suggesting that human neuroblastoma cell lines (continuous passageable cell lines) and human induced pluripotent stem cells (hiPSCs)-derived neurons (non-passageable) were among the most sensitive (Pellett, Tepp et al. 2010) (Pellett, Du et al. 2011). Neuroblastoma cell lines are easy to maintain and inexpensive in comparison to stem cell-derived neurons but as a cancerous cell line, it is suggested, that the cells may have altered gene expression that could potentially affect botulinum toxin sensitivity (Pellet 2013). There is extensive research into the capabilities of Neuro-2a (mouse) and PC12 (rat) cell lines as a suitable candidate for a cell-based assay (Schiavo, Shone et al. 1993) (Yowler, Kensinger et al. 2002) (Benatar, Willison et al. 1997), but results demonstrated that these cells are relatively insensitive; with large amounts of BoNTs required to induce SNARE cleavage.

Stem cell-derived neurons are also sensitive (Whitemarsh, Strathman et al. 2012); and although slightly more costly, they demonstrate less genetic variability as the cells are not derived from a cancerous cell line. The differentiation protocols for stem cells are typically 6 weeks and are extensive in comparison to the continuous cell line differentiation (Pellet 2013) but should yield better sensitivity to toxin to the typically mixed populations cultivated as a result of neuroblastoma differentiation.

2.3.1 Selection of Neuroblastoma Cell Lines

Three neuroblastoma cell lines were selected for screening; IMR-32, BE(2)-C and SH-SY5Y (see chapter 3 for cell line information). These lines were selected on the basis of comparable characteristics to those of the SiMa cells known to be sensitive to toxin. Neuroblastoma cell lines express neuronal properties; neural stem cell markers and tend to have an adrenergic phenotype (Mahller, Williams et al. 2009). SiMa cells, which have an adrenergic phenotype, have been demonstrated to have comparable toxin sensitivity to that of the mouse LD50

assay (Fernandez-Salas, Wang et al. 2012). Adrenergic neurons release adrenaline and noradrenaline neurotransmitters, and are abundant in the peripheral nervous system. These motor neurons are affected by tetanus toxin, (Stockel, Schwab et al. 1975) a clostridial neurotoxin similar to botulinum neurotoxin. BoNT-B, D, F and G target VAMP/synaptobrevin at the neuromuscular junction, similarly to tetanus toxin (Montecucco and Schiavo 1994) and could provide comparable sensitivity if trialled with BoNT-A dependant on SNAP-25 expression.

When considering the proven sensitivity of adrenergic neurons, a contributing factor to the selection of the neuroblastoma cell lines SH-SY-5Y, IMR-32 and BE(2)-C for screening was the high expression of the amino acid tyrosine hydroxylase (TH) (Ciccarone, Spengler et al. 1989). TH is a precursor key enzyme for three neurotransmitters collectively referred to as catecholamines (Bear, et al, 2016). Expression of TH indicates the cellular capability of producing the neurotransmitters; dopamine, noradrenaline and adrenaline. Adrenergic neurons are shown to be sensitive to toxin and this project will analyse the sensitivity of dopaminergic neurons, as their sensitivity may be comparable. Catecholaminergic neurons are found in the central and autonomic nervous system and all express TH. Papers by West (et al, 1977) and Ciccarone (et al, 1989) indicate that the three selected cell lines express TH, indicating a neuronal lineage that could be sensitive to toxin (West, Uki et al. 1977) (Ciccarone, Spengler et al. 1989).

Many established neuroblastoma cell lines have 3 morphological variants; neuroblastic (N), intermediate (I) and flat or substrate adherent (S) (Ciccarone, Spengler et al. 1989) (Ross, Spengler et al. 1995). Marker enzyme analysis presented by Ciccarone (et al, 1989) indicated that all N-type and I-type clones contained TH, hence the selection of the three neuroblastoma cell lines. BE(2)-C is I-type, SH-SY5Y is N-type and the IMR-32 cell line is a mixed population of N-type and S-type cells (Begaud-Grimaud, Battu et al. 2007).

2.3.2 Selection of Neural Stem Cell (hNSC) cell line

One commercially available neural stem cell line was selected for screening, that allows for continuous passaging of human neural stem cells, and with neural lineage, the cells can be differentiated towards motor neuron characteristics with a specific differentiation protocol. These cells may present the advantages of the use of human neural stem cells without the limitations of the cancerous cell lines. The cells are provided by Applied Stem Cell and are induced pluripotent stem cells derived from fibroblasts induced to form human neural stem cells. The hNSCs were derived using a dual SMAD inhibition methodology described by Chambers (et al, 2009) which shows that inhibiting SMAD with Noggin and SB431542 induces neural conversion (Chambers, Fasano et al. 2009). This paper also indicated that neural stem cells can then be differentiated to midbrain and spinal dopamine motoneurons, which could be sensitive to BoNTs.

2.3.3 Selection of iPSC-derived cell lines

The ReproCell ACH cells were selected as they are cholinergic neuron progenitors. Cholinergic neurons use acetylcholine, the neurotransmitter targeted by botulinum neurotoxins, as their primary transmitter. Botulinum toxin type-A is targeted highly specifically to cholinergic synapses (Dressler and Adib Saberi 2005). If the cells secrete acetylcholine, it makes them an interesting cell line to assess in the approach to testing botulinum toxin activity and potency. The ReproCell DA cell line are dopaminergic, and as discussed previously, dopaminergic neurons are catecholaminergic neurons (Bear, et al, 2016), similarly to adrenergic neurons, which have been shown to have high levels of TH and demonstrated toxin sensitivity like the SiMa cell line for example (Fernandez-Salas, Wang et al. 2012).

The iCell neurons are a mix of gamma aminoisobutyric acidergic (GABAergic) and glutamatergic neurons; two of the main neurotransmitters in the central nervous system (CNS). Most CNS synapses are mediated by the amino acids glutamate, glycine or gamma-aminobutyric (GABA). Glutamate neurotransmitter is abundant in all neurons and GABA is specifically made by GABAergic neurons. Glutamate is the major excitatory transmitter in the CNS; also present in the peripheral nervous system, and GABA mediates most synaptic inhibition in the

brain (Bear, et al, 2016). Having a combination of excitatory and inhibitory neurons replicates *in vivo* networks of synaptic function and this cell line could be effective in the detection and assessment of toxin activity. It has also been shown by Whitemarsh (et al, 2012) that this cell line is sensitive to BoNT-A.

Cell Line	Supplier	Type
iCell Neurons	Cellular Dynamics, USA	Commercial Human Differentiated iPSC-derived Neurons
ReproCell ACH cells	ReproCell, Japan	
ReproCell DA cells	ReproCell, Japan	
BE(2)-C	DSMZ, Germany	Human Neuroblastoma Cell Lines (passageable)
IMR-32	DSMZ, Germany	
SH-SY5Y	DSMZ, Germany	
Human Neural Stem Cells	Applied Stem Cell	Commercial Human Neural Stem Cells (passageable)

Table 2.1: Cell Lines Selected for Screening. *Three hiPSC-derived cell lines, three neuroblastoma cell lines and one neural stem cell line defined in table 2.1 were selected for screening based on the literature review for suitability.*

2.3.4 Approach to Cell Line Screening

A systematic approach to analysis of cell line suitability for an *in vitro* alternative for the potency testing of BoNT-A is laid out in the flow chart shown below in figure 2.1. The suitability of a cell line can be defined by four criteria described in table 2.2 below.

	Criteria
1	Ease of Culturing cells and Growth
2	Expression of SNAP-25
3	Sensitivity to Toxin
4	Expression of Toxin Receptors

Table 2.2: Defined Criteria in the Cell Line Selection. *Four defining criteria in the approach to selecting a sensitive cell line for an in vitro cell-based assay.*

Stop/no-go points	Proceed to next Criteria
Culture is time consuming, cells do not proliferate well	Culture is simple, cells grow well Proceed to expression of SNAP-25 analysis
Cells do not express SNAP-25	SNAP-25 is expressed and detected Proceed to toxin sensitivity analysis
Cells are not sensitive to toxin; cleaved and uncleaved sNAP-25 cannot be differentiated	Cleaved and uncleaved SNAP-25 detected, dose response to toxin Proceed to expression of receptors for further justification data collection

Table 2.3: Stop/ no-go Points in the Approach to Selecting a Suitable Cell Line. *Following the order of the criteria in table 2.3, each criterion has a stop point, or an allowance to proceed with a cell line that meets the criteria specifications.*

Assessing the growth and ease of culture as the primary step in cell line selection is necessary when applying its use in everyday routine laboratory work. The cells will be required for use in testing on a weekly basis so will need to be easy to culture, with low maintenance with regard to media preparation and proliferation/ passaging of cells. The cells will need to be reliable and robust, with marginal variability as repeatability of results and cell culture conditions are necessary for a GMP-compliant assay for pharmaceutical testing (criteria 1). To analyse the expression of SNAP-25 is an important consideration for cell line selection; without SNAP-25 expression, toxin activity cannot be determined (criteria 2). This is an essential requirement for this test, as is criteria 3; sensitivity to toxin. Sensitivity, in these terms, refers to the cell lines ability to survive during toxin incubation, uptake the toxin into the cytosol and allow toxin translocation to result in subsequent cleavage of SNAP-25. The cells should be sensitive to low doses of toxin, and SNAP-25 cleavage reflecting toxin activity should be detectable at levels comparable to potency calculation with the mouse LD50. Criteria 4 refers to the toxin receptor expression of the cell line, with particular reference to SV2 proteins enabling the binding of toxin. This criterion can be paired with criteria 3 as cell line sensitivity to toxin is theoretically enabled by receptor mediated endocytosis, suggesting that if results indicate toxin sensitivity in the experiments investigating criteria 3, that the subsequent results for receptor expression should be complimentary and confirm specificity.

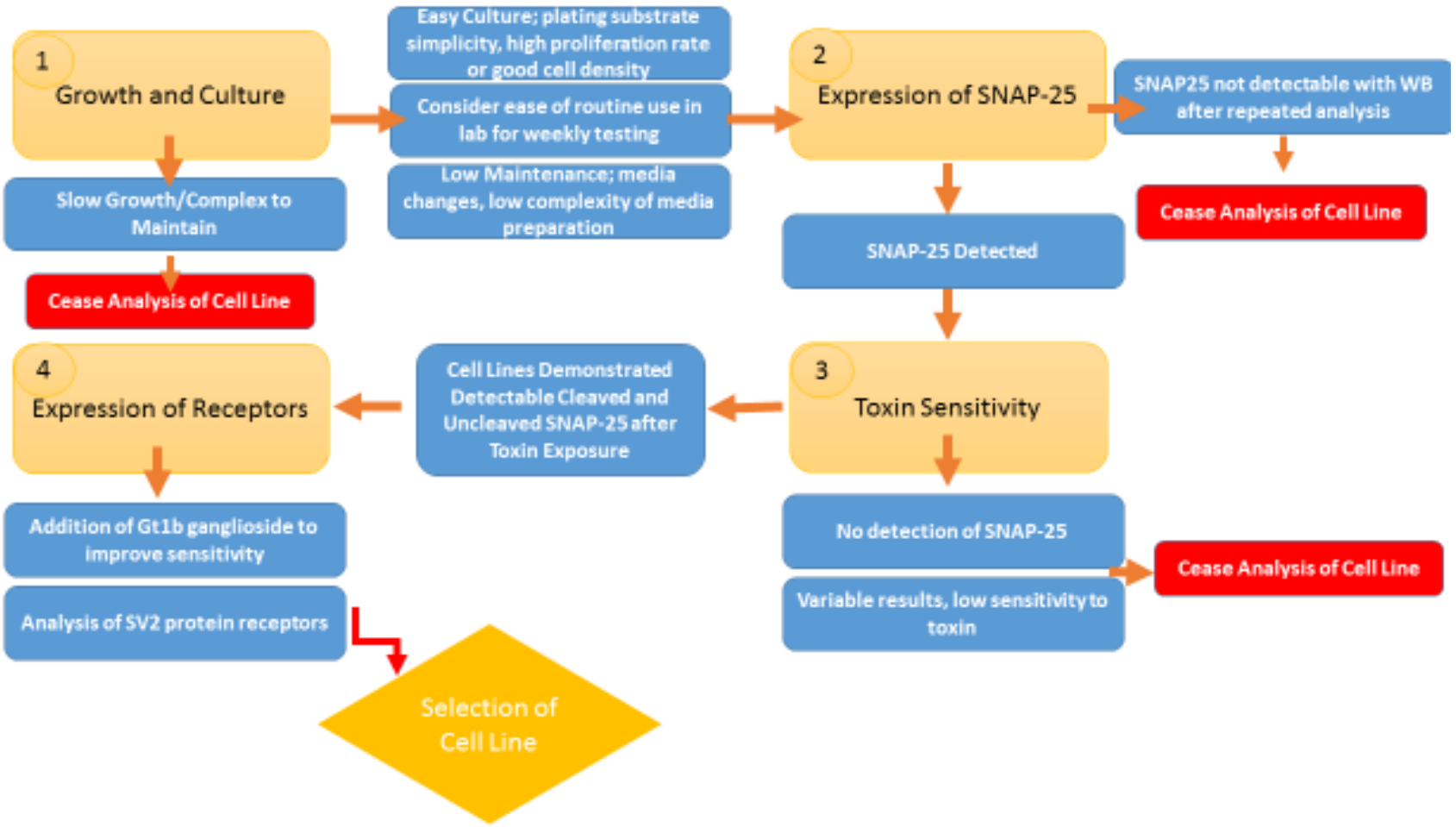


Figure 2.1: Diagram Representation of Cell Screening Procedure. *Flow Chart illustrating the experimental approach to cell line selection, based on the requirements of an in vitro alternative for industry.*

2.4 Selecting and Optimising a Detection Method

A detection method for the quantification of toxin activity and thus being able to determine the toxin potency is an essential element of developing a cell-based assay for the pharmaceutical batch release testing of Botulinum toxin, as required for this project. Assays have been submitted to the FDA for approval in recent months, all of which use a patented antibody detection system to detect toxin activity and the cleavage of SNAP-25. This project aims to trial the antibody detection methods detailed in table 2.4 below to determine suitability for a GMP pharmaceutical cell-based assay, alongside other potential sensitive methods that do not require the use of antibody detection for a novel and unique approach. (see Chapter 7 for detailed protocols for each method).

Detection Method	Benefits/Reasons for Selection
Western Blot	Used predominantly in literature for cell sensitivity screening- antibody detection of SNAP-25 is sensitive. Proven to detect both cleaved and uncleaved SNAP-25, and will be used to assess expression of SNAP-25, and subsequent toxin sensitivity (determined by SNARE cleavage) in each cell line selected for screening.
Microchip Electrophoresis (MCE)	Uses fluorescence to detect proteins in samples. Similar to a Western Blot, but MCE uses very small volumes of sample at high throughput- Ideal if using an expensive cell line for example.
Mass Spectrometry	Sensitive method, able to detect synthetic SNAP-25 and define serotypes of BoNT. This is a non-antibody approach detection method, which will be novel in the field.

Table 2.4: Potential Detection Methods for a Cell-Based Assay. *These methods were preliminary outlined for the potential to be sensitive and adaptable for a pharmaceutical cell-based assay.*

2.4.1 Western Blot

Western Blot methodology was selected as it is regularly used for the detection of proteins in a cell lysate and is an analytical technique that can separate proteins by molecular weight. This project aims to look at detecting the differing cleaved and uncleaved SNAP-25 (24kDa and 25kDa respectively) and separation on a gel should allow for the distinction between cleaved and uncleaved. Proteins are transferred to a membrane, then incubated with primary and secondary antibodies specific to the protein of interest; anti-SNAP-25 for this project. This methodology therefore uses antibody detection; elements of the use of antibodies methodology for BoNT-A activity detection have been patented so this will need to be taken into consideration. Analysing lysate for SNAP-25 alongside a house-keeping protein such as Beta-Actin, allows for quantification of protein band intensity of detected SNAP-25 to determine toxin activity.

2.4.2 Microchip Electrophoresis (MCE)

This method provides the opportunity to separate proteins by molecular weight, much like a Western Blot. It is an analytical technique that incorporates droplet-based microfluidics providing a system that is high-throughput with the capabilities to load several samples at the same time and the sample volume required, compared to that used in a Western Blot analysis, is significantly reduced. The migration of nano-litre droplets of sample through a gel in the chip allows separation and identification of proteins. Sample is loaded into channels filled with separation buffer and wire electrodes are connected; the methodology, described in detail in Chapter 7, allows for the generation of three droplets, i.e. three replicates per sample, which migrate performing electrophoresis separation. Fluorescein dye solution is added allowing the droplet intensity analysis with a fluorescent microscope. The reduction in sample required and high-throughput is attractive to this project as it could potentially allow for the parallel testing of several samples, with three replicates generated per analysis/sample; ideal for demanding testing requirements in the company.

2.4.3 Mass Spectrometry

Mass Spectrometry is used as a technique for the identification of proteins, determining specificity through mass analysis. Sample is run through a mass spectrometer as charged droplets and these droplets pass down a pressure gradient and are analysed by molecular mass. Utilising mass spectrometry as the detection method in a cell-based assay for the testing of Botulinum toxin introduces a novel concept to the assay methodology. It eliminates the use of antibody-based detection and can potentially detect low abundance proteins in minimal sample volumes. This would be an ideal candidate if an expensive cell line was selected, for example, as it would potentially considerably reduce the quantity of sample, and subsequently the concentration of protein (reflected also in the number of cells required per sample) required for successful analysis of SNAP-25 and toxin activity; detecting both cleaved and uncleaved isoforms.

2.4.4 Approach to Screening Detection Method Suitability

Tables 2.5 and 2.6 below define the criteria and stop/no-go points which apply to each of the detection methods for the screening process. Analysis of cell line suitability begins with Western Blot analysis, and once the optimum concentrations of samples are obtained, the mass spectrometry analysis and optimisation of the microchip methodology will be run in parallel. There are several contributing factors that will allow proof of concept, or indication as to when to eliminate the method as a potential candidate shown in table 2.6 below.

Criteria	Specifications/Requirements
1	Detection of Whole SNAP-25
2	Detection and Distinction between Cleaved and Uncleaved SNAP-25
3	Repeatable and Reliable Results with the Selected Cell Line
4	Reliability of Quantification and Determination of Potency

Table 2.5: Defined Criteria in the Detection Method Selection. *Four defining criteria for selecting a detection method for a cell-based assay.*

Detection Methods	Stop/no-go points
Western Blot	Detection of SNAP-25 not possible
Mass Spectrometry	Distinction between cleaved and uncleaved SNAP-25 not possible
Microchip Electrophoresis	GMP compliant quantification method not feasible

Table 2.6: Stop/ no-go Points in the Approach to Selecting a Suitable Detection Method. *Each of the stop/ no-go points in the table apply to each of the detection methods concurrently.*

Firstly, criteria 1 specifies that the method must detect whole SNAP-25, an indication that the method can potentially be optimised for use in an *in vitro* assay. Without the successful detection of SNAP-25, a low abundance protein, the methodology is unusable. The secondary stage (criteria 2) is to ensure the detection and separation of both cleaved ($SNAP_{197}$) and uncleaved ($SNAP_{206}$) SNAP-25 in cell samples after toxin incubation. The detection of both forms of SNAP-25 is essential for the quantification of toxin activity. If the detection method successfully distinguishes cleaved and uncleaved SNAP-25 criteria 3 states that it must be trialled, and work successfully with the selected cell line. The cell line will be selected based on the criteria described previously, and the most suitable candidate will provide reliable and consistent results, which must be replicated on the selected detection method. Western Blot will be used to screen the cell lines for suitability and sensitivity to Botulinum toxin type-A, and the results will indicate the best cell line candidate for this project. Western blot is a method well documented for the identification of proteins in cell lysate samples, deeming it a suitable method for the screening of cell line sensitivity and subsequent selection. The method however is susceptible to variation, with complex elements to the protocol and opportunity for misinterpretation in quantification. The method could not be QC-validated for use in a GMP compliant routine assay method for the *in vitro* alternative assay for the testing of botulinum toxin. Therefore, using toxin treated samples of the selected cell line, the potential detection method should be functional and reliably detect toxin activity. The method must be robust and repeatable, be utilisable in a working laboratory and most importantly be sensitive enough to detect toxin at doses and levels comparable to those calculated with the mouse LD50. Criteria 4 signifies the importance of ensuring that the selected detection method, and the complete assay, are appropriate for validation and use relating to GMP compliancy and ICH guidelines described in sections 2.5 and 2.6.

The diagram in figure 2.2 indicates the approach to screening each detection method, with the decision to discard the methods if detection of SNAP-25 is not achievable or some criteria are not met.

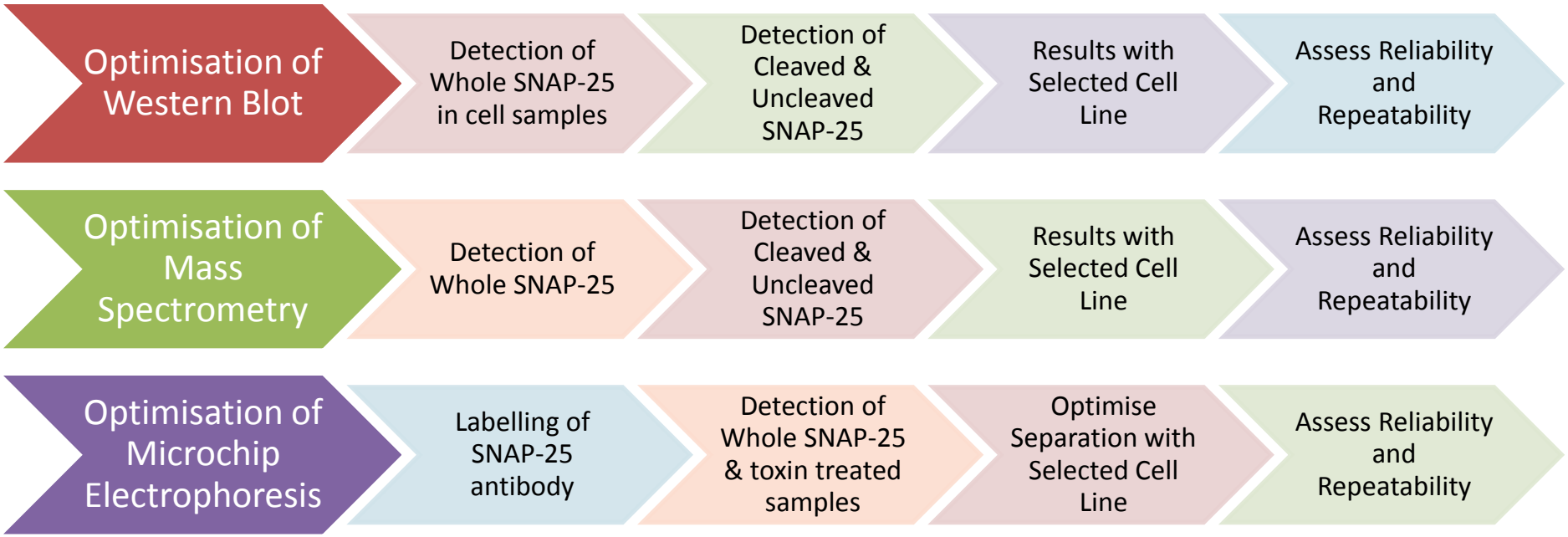


Figure 2.2: Diagram Representation of the Detection Method Screening Procedure. *Flow diagram demonstrating the approach to screening selected detection methods. Detection methods will be screened in parallel and each method have designated stop/no-go points, outlined in table 2.6.*

2.5 Regulatory Submission Preparation

On selection of a suitable cell line and a detection method, the complete assay will be subject to validation for regulatory submission. There are several steps to the validation process to ensure that the cell-based alternative *in vitro* assay is a suitable candidate as a replacement for the currently approved method. The International Conference on Harmonisation (ICH) provided standards and guidelines to consider when designing an analytical procedure for submission, specifically for tests and procedures relating to the release of drug substances or drug products. These are disclosed in table 2.7 below.

Criteria	Definition	
Accuracy	The closeness of agreement between the value which is accepted as a true or reference value and the value found	
Precision	Closeness of agreement between series of measurements obtained from multiple sampling of the same sample under same conditions.	
	Repeatability	Under same operating conditions over short interval
	Intermediate Precision	Within-laboratory variations; different days, analysts, equipment.
	Reproducibility	Precisions between laboratories (collaborative)
Detection Limit	The lowest amount of analyte detectable in a sample	
Quantitation Limit	The lowest amount of analyte quantifiable with suitable precision and accuracy.	
Linearity	Ability (within a given range) to obtain test results which are directly proportional to the concentration of analyte	
Range	Interval between the upper and lower concentration of analyte for which it has been demonstrated that there is a suitable level of accuracy, precision and linearity	
Robustness	Measure of its capacity to remain unaffected by small variations in method parameters; an indication of its reliability during normal usage	

***Table 2.7: The ICH Guidelines for the Validation of Analytical Procedures (2005)**. These considerations must be adhered to in the validation process of an alternative in vitro assay for the testing of Botulinum Toxin.*

All of the factors described above are a necessity for the successful submission of an analytical method with reference to testing drug products for release. The accuracy is paramount when considering the powerful and possibly detrimental effect of Botulinum toxin and accurately determining its potency before release is of upmost importance. To repeatedly test in a working laboratory environment, the assay used must be reliable in its results, as it will be relied upon on a weekly basis, testing several samples. For an alternative to be accepted in place of the robust and 'gold-standard' mouse bioassay, the proposed methodology must be considerate of all ICH guidelines.

2.6 Industry Expectations of a Cell-Based Assay for BoNT-A Potency Testing

In 2008 the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) held a workshop meeting together with the NICEATM and ECVAM (National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods and the European Centre for the Validation of Alternative Methods) reporting on alternative methods to refine, reduce or replace the mouse LD50 assay for Botulinum toxin testing. The panellists of the meeting consisted of specialists in the field of botulinum toxin testing and detection and several requirements for a replacement assay were specified.

As reported from this meeting, a satisfactory test must be able to differentiate between the seven known serotypes of toxin, function reliably with diverse samples, be capable of detecting low levels in clinical specimens and be easily replicated and reproducible in different laboratory settings. Sensitivity, specificity, reproducibility and robustness are key elements for consideration when designing a replacement assay. A potency assay must determine the dose of the final product, compare relative activities of different batches or lots and as BoNT activity is dependent on three functional domains within the protein; the assay must account for the activity of all domains (binding and internalisation, translocation and enzymatic activity). The mouse LD50 assay accurately determines clinical dose levels and measures activity of each of the

three domains successfully and is the 'gold standard' to potency testing for Botulinum toxin.

The 2008 Scientific meeting concluded that several factors should be considered when attempting to produce a cell-based assay alternative to the LD50, described below;

- The assay needs to be as sensitive as the mouse LD50 showing a reproducible correlation between activity and mouse LD50 units.
- It should detect all toxin sub-types. For BoNT-A the assay should be able to detect all four subtypes A1-A4 with comparable sensitivity.
- The sample media should not affect the sensitivity.
- It should take less time than the mouse LD50 to conduct.
- The cost should not be prohibitive.
- The results should be reproducible in every laboratory setting.

Chapter 3: Culture and Growth of Cell Lines

3.1 Introduction

As described in Chapter 2; project plan, the culturing and maintenance of a cell line to be selected for routine pharmaceutical testing is an important consideration. In an industry environment, samples can be sent by clients weekly, or sometimes as a last-minute test requirement, so an assay system that is quick to set up is a necessity. If samples are tested, and the results are inconclusive, and a re-test is required, it would be poor practise to insist that the client wait several weeks, as an example, for the cells to be available for use. A cell line that is problematic and hard to culture, has issues with adherence, slow proliferation or requires a complex and lengthy differentiation protocol to ensure a sensitive cell population for use would not be an ideal candidate for routine assay testing. Currently, the mouse LD50 test requires the mice to have a four-day habituation in-lab before subject to testing, and extra mice are always available to cover the possibility of any errors. Animals can be reordered quickly for repeat testing but still require the four-day habituation, further delaying the provision of results to the clients. Cell lines will relieve this restriction; if a cell line that is easy to maintain can be shown to be suitable for the use in testing botulinum toxin type-A, then cells should be readily available for use every working day.

There are principles and standards, defined in table 3.1 below, that should be adhered to in the determination of proceeding with a cell line for the analysis of SNAP-25 (criteria 2 defined in the project plan) through analysis of ease of culture and growth and these criteria are important considerations for a cell lines overall suitability for this project. The proliferation rate (time and number of cells) of each cell line and the maintenance procedure for culturing the cells will be discussed. The ease of culture media preparation is a factor for consideration; several members of staff will perform the cell-based assay on a routine basis so variability in the make-up of the maintenance media and supplements for a selected cell line should, ideally, be minimal.

Principle/Standard	Cell Line Observations	Action
Proliferation	Cells proliferate well, efficient doubling time and available cell number	Consider cell line for criteria 2 analysis (expression of SNAP-25)
	Cells are slow growing, low cell number availability	Eliminate cell line as a suitable candidate
Culture Maintenance	Cell maintenance is simple; media changes fit in with a weekly work schedule, media make-up has minimal risk of variation	Consider cell line for criteria 2 analysis (expression of SNAP-25)
	Maintenance is complex, frequent media changes required, complex media make-up which increases error risk	Eliminate cell line as a suitable candidate
Repeatability of Culture conditions	Cell line consistently grows well, with expected morphology and is reliable to culture with minimal contaminations or errors	Consider cell line for criteria 2 analysis (expression of SNAP-25)
	Cell culture is variable, high cell death, no adherence/differing morphology	Eliminate cell line as a suitable candidate

Table 3.1: Principles and Requirements for Consideration for Criteria 1; Ease of Culture and Growth. *The tabled criteria are considerations when analysing the culture and growth of each of the selected cell lines.*

3.1.1 Defining Criteria in Analysing Culture and Growth of Cell Lines

When analysing the suitability of a cell line in relation to the ease of culture, the proliferation rate of the cells is a primary consideration. The cells are required to be available for use promptly, and a cell line that takes weeks to generate enough viable cells for testing conditions would not be suitable. Cells will be maintained consistently on a daily basis in a working laboratory environment but a quick 'start-up' would be an ideal characteristic.

The ease of culture maintenance is also a requirement, with the number of media changes necessary before the cells are suitable for use and the complexity of the make-up of the maintenance media to be reflected. As previously mentioned, several laboratory technicians will be carrying out the assay in a working environment and this enables the risk of variability and error. A maintenance media that requires minimal preparation reduces the risk of error in the make-up of the media, and a cell line that requires minimal media changes minimises the exposure of the cells to contamination or error. Aseptic technique is enforced in GMP laboratories and extensive training is provided to ensure that all staff are fully competent, thus reducing the risk of error further, but ensuring that the maintenance of the cells provides minimal risk of variability is a vital consideration.

The last defined criteria in the approach to assessing cell line ease of culture and growth is the repeatability of the culture conditions. The selected cell line must consistently grow well in culture, provide a consistent number of viable cells when required and this must be repeated in each experimental condition or test and with either each vial of cells used, or each differentiated cell population. If a cell line does not adhere to culture-ware as it should and has a different morphology than expected, thus requiring extensive protocols to ensure proper maintenance and cell growth, or has a high cell death, with or without application of differentiation reagents it would not be suitable for selection.

To summarise, an ideal cell line should grow well, and proliferate efficiently, the protocol should be amenable to differing environments or perpetrators and the cell line should be repeatable and predictable in its culture requirements. Each cell line will be scrutinised using the criteria defined in table 3.1 and discarded for suitability if the culture is complex, unsuccessful or too problematic.

A study by Beske (et al, 2015) provided data that suggests that GABAergic embryonic stem cell-derived neurons (ESNs) are sensitive to Botulinum toxin type-A, demonstrating that BoNT-A blocks synaptic neurotransmission between glutamatergic and GABAergic ESNs, confirming that these cell types replicate *in vivo* clinical botulism effects (Beske, Scheeler et al. 2015). This illustrates that synaptic function could be an important factor for consideration when selecting a sensitive cell line, ensuring synaptic response and maximum potential sensitivity to BoNT-A.

3.1.2 Neuroblastoma Cell Lines

Neuroblastoma is a rare cancer, which develops from nerve cells, mostly affecting young children. This cancer firstly develops in the adrenal glands and can easily spread to the bones and liver. These cell lines, derived from these types of tumours, were selected for screening for toxin sensitivity as the SiMa neuroblastoma cell line has been published to demonstrate excellent sensitivity, leading to it being used in an approved cell-based assay (Fernandez-Salas, Wang et al. 2012). SiMa cells have an adrenergic phenotype, and express tyrosine hydroxylase (TH); documented to determine adrenergic phenotype as a catecholamine similarly to dopaminergic phenotype. The SH-SY5Y, BE(2)-C and IMR-32 were selected based on the comparable characteristic of the SiMa line; expression of TH. For this project, it was interesting to look at different strains of neuroblastoma cells to see if these could also be as or even more sensitive.

3.1.2.1 BE(2)-C Neuroblastoma

The BE(2)-C cell line was purchased from Public Health England. This cell line is a clonal sub-line of SK-N-BE(2) which was isolated from bone marrow from a patient with disseminated neuroblastoma in 1972.The data sheet indicates that the cells are multipotential with regard to neural enzyme expression and display a high capacity to convert tyrosine to dopamine. The cells grow in aggregates with short neurite extension. Figure 3.1 shows images of the cells captured at both 24-hours and 48-hours post-seeding; images provided by the supplier of the cell line.

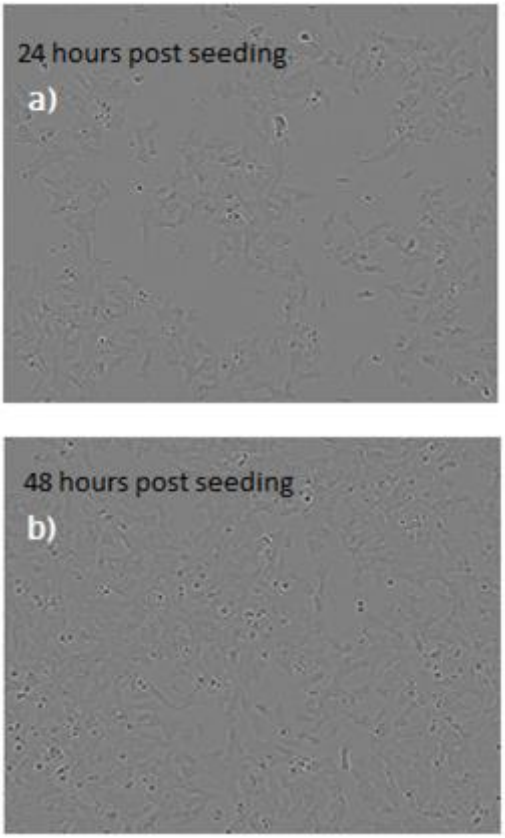


Figure 3.1: BE(2)-C Cell Line; Images supplied by Public Health England General Culture Collection*. (BE(2)-C data sheet). Phase contrast images of cells* ***(a)*** *24-hours post plating and* ***(b)*** *48-hours post plating. The cells in image b) are close to requiring passaging.*

3.1.2.2 SH-SY5Y Neuroblastoma

The SH-SY5Y cells were purchased from Public Health England. This cell line is thrice-cloned sub-line of SK-N-SH and has dopamine-beta-hydroxylase activity, expresses the neurotransmitter GABA and should be sensitive to toxin based on these tendencies (Beske, Scheeler et al. 2015). It is a supplier recommendation to verify specific characteristics of this cell line routinely as the loss of neuronal characteristics can occur with increasing passage numbers so it is the intention to keep this as low as possible whilst providing a substantial stock. Figure 3.2 below shows SH-SY5Y cells 24-hours and 48-hours post-seeding, images provided by the supplier of the cell line.

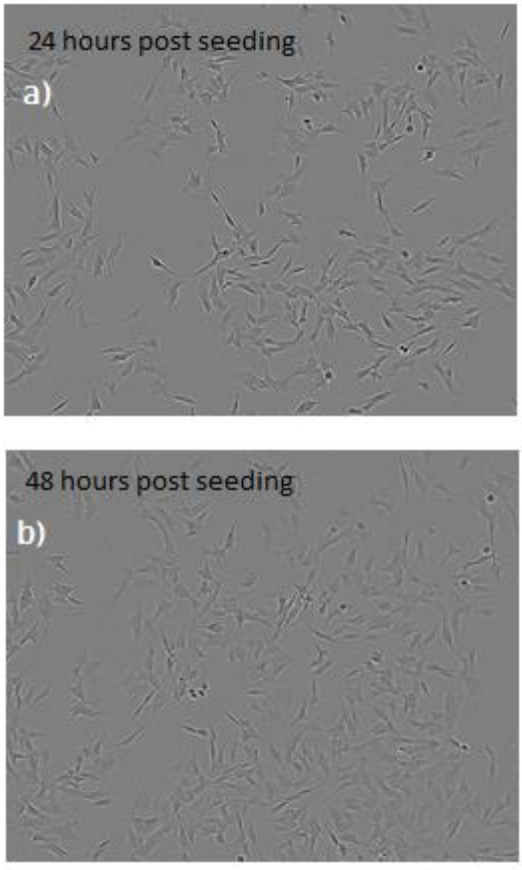


Figure 3.2: SH-SY5Y Cell Line; Images supplied by Public Health England General Culture Collection*. (SH-SY5Y data sheet).* ***(a)*** *SH-SY5Y cells 24 hours post plating, lower cell density is recommended compared to BE(2)-C cells, allowing for dendrite extension.* ***(b)*** *SH-SY5Y cells 48 hours after plating, cells have proliferated but are not ready for passaging.*

3.1.2.3 IMR-32 Neuroblastoma

The IMR-32 cells were purchased from DSMZ. This cell line was established from an abdominal mass in 1967 from a patient with neuroblastoma. The culture is a mixture of two morphologically distinct cell types, a small neuroblast-like cell and the other a large hyaline fibroblast. (Tumilowicz, Nichols et al. 1970). IMR-32 neuroblastoma cell line is extensively used in publications in research for Alzheimer's Disease, given its susceptibility to neuronal differentiation (Neill, Hughes et al. 1994) (Yan, Bienkowski et al. 1999). Figure 3.3 shows an image provided by the cell line supplier of the expected morphology of the IMR-32 cell line 48-hours after initial plating.

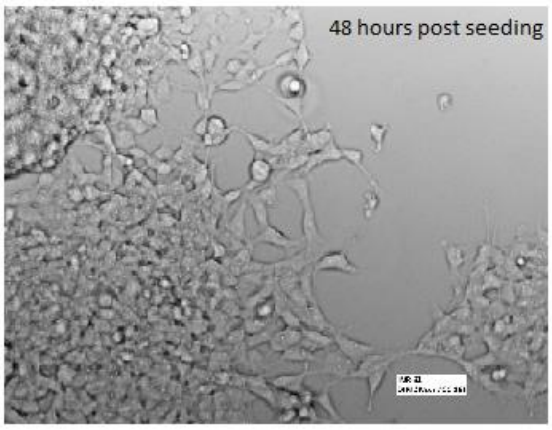


Figure 3.3: IMR-32 Cell Line; Image supplied by DSMZ. *(data sheet). IMR-32 cells 48 hours post seeding. Phase contrast image of IMR-32 cells. Cells have adhered to the plate in a monolayer form, but can proliferate for approximately a further 48 hours before passaging.*

3.1.3 iPSC-derived cell lines

Neurons derived from human induced pluripotent stem cells (iPSCs) are more sensitive to botulinum toxin in comparison to primary neurons (Whitemarsh, Strathman et al. 2012) and ensure that a human cell line can be used, as opposed to sourcing primary cells from animals in an intended animal replacement cell-based assay. The human iPSC-derived cell lines selected for screening are described below.

3.1.3.1 Human Neural Stem Cells

These human neural stem cells (hNSCs) are supplied by Applied Stem Cell and are derived from iPSCs, which in turn were derived from human foreskin fibroblasts induced to form neural epithelial rosettes. They are low passage neural stem cells that can be further differentiated to the neural cell type of choice. Figure 3.4 shows the expected morphology of the hNSCs, provided by Applied Stem Cell.

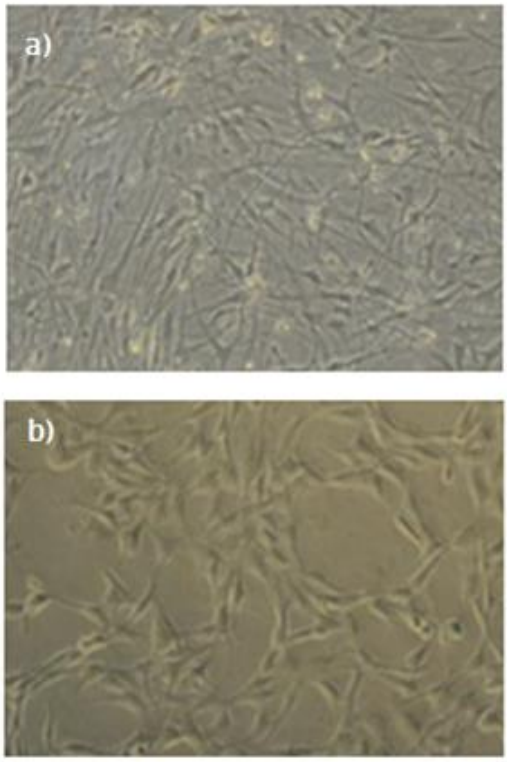


Figure 3.4: Human Neural Stem Cells; Images supplied by Applied Stem Cell*. (data sheet for maintenance of human neural stem cells). 10x phase contrast images of cells* ***(a)*** *cells at a density recommended for passaging to allow for further proliferation* ***(b)*** *initial and optimal plating density (6 well plate, 1 x* 10^6*).*

3.1.3.2 ReproCell ACH and DA cells

Also commercially produced differentiated cell lines, the DA and ACH cell lines were purchased as a kit, provided by ReproCell. This includes the plate coating solution, media and supplement for the successful maintenance of their products. The ACH cells are cholinergic neuron progenitors derived from human iPS cells and the DA are dopaminergic neuron progenitors derived from human iPS cells, both viable for assay use on day 19 of culture. The project had a limited supply of these cell lines, two vials were purchased for preliminary analysis of the cells before committing to replicate experiments. Images of the DA cell line are shown in figure 3.5 below, 24-hours after plating and the differentiation of the cells after 14 days of maturation.

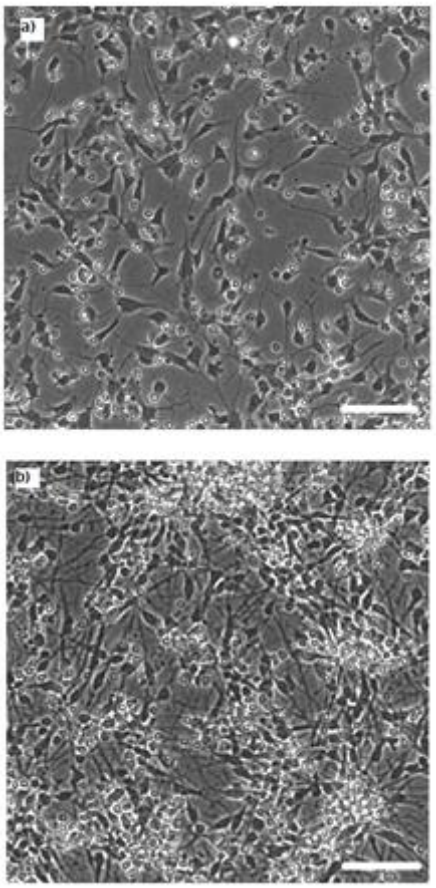


Figure 3.5: ReproCell DA Cells, Images supplied by ReproCell. *(data sheet for DA cell line kit).* ***(a)*** *24 hours after plating* ***(b)*** *14 days after plating cells in maturation medium differentiate fully into dopaminergic neurons. Images for the ACH cell line were no longer available from the supplier website. Scale bar 100µm.*

3.1.3.3 iCell Neurons

Figure 3.6 shows images captured of iCell neurons, provided by Cellular Dynamics, which are induced pluripotent stem cells (iPSCs) differentiated into human cerebral cortical neurons. Cellular Dynamics claim a 95% population of glutamatergic and GABAergic neurons. Supporting documents and data from the supplier suggests long term viability and rapid formation of functional synapses; which is suggested to be vital for a 'true' representation of cellular behaviour in vivo in reaction to botulinum neurotoxins (McNutt, Celver et al. 2011) (Beske, Bradford et al. 2016) (Beske, Scheeler et al. 2015). Although this is not defined as a criteria requirement alongside SNAP-25 expression, any additional attributes, such as functional synapses, that will be beneficial in relation to the cells line sensitivity to botulinum toxin will be considered and tested and help this project determine the best cell line candidate.

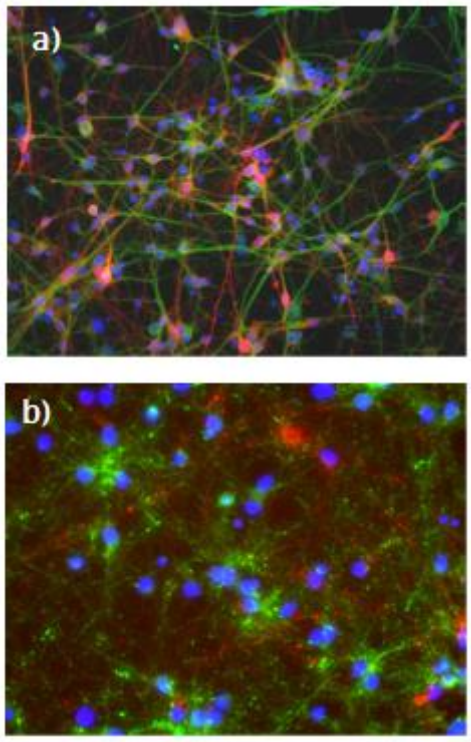


Figure 3.6: iCell Neuron Staining; Images supplied by Cellular Dynamics. *iCell neuron maintenance guide.* ***(a)*** *GABA (red), MAP2 (green) and Hoechst (blue) staining for GABAergic neurons.* ***(b)*** *vGLUT2 (red), vGAT (green) and Hoechst (blue) staining for glutamatergic neurons.*

Each of the selected cell lines were screened according to criteria 1; ease of culture and growth of cell lines and progressed to criteria 2 if suitable. The complexity of the maintenance methodology and time between passages or growth time are considerations at this stage of screening. The selected cell line needs to be reliable, and provide viable testing conditions for routine testing, without complex, time-consuming protocols to ensure repeatable cell culture conditions. The number of viable cells upon receipt from supplier and general culturing methods i.e. complexity of making media, considering variables when relating to a repeatable, GMP compliant cell culture method are also considered in the selection of a suitable cell line.

3.2 Methods – Culture and Growth of Cell Lines

This section describes the cell culturing methods used as part of the cell based assay development with specific focus on the ease of culture and growth of each cell line. These factors are primary considerations when selecting an appropriate cell line for use in commerical testing, with ease of culture contributing to the assay turn-around from plating to results of testing, therefore making economic impact. Problematic cells, with extensive culture routines to ensure proliferation and cell survival would be ruled out if alternatives are easier to handle.

Cell lines were purchased from the suppliers indicated in the Chapter 3, section 3.1 introduction, and on delivery, stored in liquid nitrogen until required for use.

All cell culture was performed in a Category 2 laboratory in a biological safety cabinet, using asceptic technique. Good laboratory practise (GLP) standards were maintained when handling toxin, using hypochloride solution to decontaminate any opened and used vials, double gloved when handling and any pipettes used in the preparation of toxin samples were cleaned thoroughly after use. Toxin is stored in a secure refrigerated container with restricted access.

3.2.1 BE(2)-C Neuroblastoma Cell Line

Supplier: Public Health England General Cultures Lot #: 10H023

Preparing Culture Surfaces: The cells adhere to culture ware without the requirement of coating solutions.

Thawing Cells: Cells are thawed in a 37˚C water bath for approximately 2 minutes, until the cell solution has thawed and immediately transferred to 15mL centrifuge tube. 1mL of warmed maintenance media (table 3.2) is added to the cryovial to collect residue cells and transferred to the cell suspension drop-wise to minimise osmotic shock. The cells are centrifuged for 4 minutes at 300 *xg* and re-suspending the cells in 1mL of media allows for cell count with a haemocytometer.

Plating Density: 30,000 cells/cm² (750,000 cells/25cm² flask/10mL media and 60,000 cells/well on 24 well plates/500µl media))

Maintenance: Complete media changes were carried out using a 10mL graduated pipette to remove all media, without detaching the cells from the flask and replaced with 10mL fresh maintenance media (table 3.2) every 3-4 days, usually coinciding with passaging due to confluence of cells.

Passaging: To passage BE(2)-C neuroblastoma cells, all media is removed from the flask using a 10mL graduated pipette and discarded. 3mL per 25cm² flask of trypsin is added to the cells adhered to the flask, and the flask returned to the incubator for approximately 3 minutes. To assess the detachment of the cells from the flask within this incubation time the cells were observed under the microscope; the trypsin should help the cells to detach and provide a single cell suspension for cell counting. Adding 3mL of maintenance media containing serum to the dissociated cells ceases the dissociation process and the cell suspension is collected in a 15mL tube and centrifuged at 300 *xg* for 3 minutes, until a cell pellet is formed in the tube. Media and trypsin supernatant is discarded and the cell pellet re-suspended in 1mL of media, using a pipette, for the cell count. Cells are counted by mixing 10µl of cell solution with 10µl trypan blue (1:1) and counted on a haemocytometer (refer to Appendix B).

Freezing Cells: The neuroblastoma cell lines proliferate well so many cells were frozen and stored in liquid nitrogen for later use. BE(2)-C cells are frozen as 3

million cells/cryovial in 1mL of freezing medium (see table 3.2). Cells were collected as a cell pellet, as per the passaging protocol, and re-suspended in 1mL of freezing medium and stored at -80˚C overnight, before transfer to liquid nitrogen.

Solution	Components	Concentration prep 40mL	
Maintenance Media	EMEM (EBSS)	1x	16.6mL
	Hams F12	1x	16.6mL
	Non Essential Amino Acids	1%	400µl
	Glutamine	2mM	400µl
	Heat inactivated Foetal Bovine Serum (FBS)	15%	5.6mL
	Penicillin/Streptomycin	1%	400µl
Freezing Media	Maintenance Media	90%	36mL
	DMSO	10%	4mL

Table 3.2: Solutions for Culturing BE(2)-C Neuroblastoma Cell Line. See Appendices for Suppliers and Product Codes.

3.2.2 SH-SY5Y Neuroblastoma Cell Line

Supplier: Public Health England General Culture Lot #: 11C016

Preparing Culture Surfaces: The neuroblastoma cell line does not require prior coating of culture flasks or plates and the cells adhere to the surface without.

Thawing Cells: Cells are thawed in a 37˚C water bath for approximately 2 minutes- until the cell solution has thawed and immediately transferred to a 15mL centrifuge tube. Adding 1mL of warmed maintenance media (table 3.3) to the cryovial collects residue cells, and is transferred to the cell suspension dropwise to minimise cell death. The cell solution is centrifuged for 4 minutes at 300 *xg* and the supernatant discarded. Re-suspending the cells in 1mL of media allows for cell count with a haemocytometer (Appendix B).

Plating Density: 10,000 cells/cm² (250,000 cells/25cm² flask/10mL media and 20,000 cells/well on 24 well plates/500µl media)

Maintenance: Similarly to the BE(2)-C cell line, complete media changes are required every 3-4 days, which primarily coincides with the requirement for passaging of the fast expanding cell line. When required, 10mL media/flask is replaced, without detaching the adherent cells.

Passaging: To passage the SH-SY5Y cells, the protocol is the same as for BE(2)-C. Discarding the media, adding 3mL/flask trypsin and incubating dissociates the cells. Cell counts performed as described previously and plated at density of 10,000 cells/cm² (250,000 cells/flask/10mL maintenance media).

Freezing Cells: SH-SY5Y cells were collected from flasks, as described above, and the cell pellet re-suspended in 1mL freezing media (table 3.3) and stored at -80˚C overnight before transfer to liquid nitrogen.

Solution	Components	Concentrations prep 40mL	
Maintenance Media	EMEM (EBSS)	1x	16.6mL
	Hams F12	1x	16.6mL
	Non-essential amino acids	1%	400µl
	Glutamine	2mM	400µl
	Heat inactivated Foetal Bovine Serum (FBS)	15%	5.6mL
	Penicillin/Streptomycin	1%	400µl
Freezing Media	Maintenance Media	90%	36mL
	DMSO	10%	4mL

Table 3.3: Solutions for Culturing SH-SY5Y Neuroblastoma Cell Line. See Appendices for Suppliers and Product Codes.

3.2.3 IMR-32 Neuroblastoma Cell Line

Supplier: DSMZ Lot #: 7.14.12.2012

Preparing Culture Surfaces: It is recommended by the supplier not to coat the plates before culturing IMR-32 cells.

Thawing Cells: The IMR-32 cells are thawed following the same procedure as the other neuroblastoma cell lines; cells are thawed in a 37˚C water bath for approximately 2 minutes, until the cell solution has thawed completely and the cells are immediately transferred to a 15mL centrifuge tube. Adding 1mL of warmed maintenance media (table 3.4) is added to the cryovial collects residue cells, and is transferred to the cell suspension dropwise to minimise cell death. and re-suspended in 1mL fresh maintenance media. The cell solution is centrifuged for 4 minutes at 300 *xg* and the supernatant discarded. Re-suspending the cells in 1mL of media allows for cell count with a haemocytometer (Appendix B).

Plating Density: 10,000 cells/cm² (250,000/25cm² flask/10mL maintenance media and 20,000 cells/well on 24 well plates/500µl media)

Maintenance: Proliferation media is changed every 3-4 days, removing 10mL media from flasks using a graduated pipette. The cells are suspended as well as adherent, so the media is centrifuged to collect any suspended cells, and media is replaced slowly to prevent detachment of the adherent cells.

Passaging: The IMR-32 cells detach easily so there is no requirement for trypsin. A gentle stream of media from a graduated pipette will remove the cells and the media is collected in a 15mL tube and centrifuged at 300 *xg* for 3 minutes to form a cell pellet. Cells are counted with trypan blue and are typically split 1:2 after 3-4 days proliferation.

Freezing Cells: IMR-32 cells were collected from flasks, as described above, and the cell pellet re-suspended in 1mL freezing media (table 3.4) and stored at -80˚C overnight before transfer to liquid nitrogen.

Solution	Components	Concentrations prep 40mL	
Maintenance Media	RPMI 1640	78%	31.2mL
	Heat inactivated FBS	20%	8mL
	Non-essential amino acids	1%	400µl
	Penicillin/Streptomycin	1%	400µl
Freezing Media	Maintenance Media	90%	36mL
	DMSO	10%	4mL

Table 3.4: Solutions for Culturing IMR-32 Neuroblastoma Cell Line. See Appendices for Suppliers and Product Codes.

3.2.4 Human Neural Stem Cells

Supplier: Applied Stem Cell

Preparing Culture Surfaces: Frozen Geltrex (Thermo Fisher #A1413302) stock (12mg/mL) is thawed and diluted in maintenance media to prepare coating solution (see table 3.5). On 24-well plates, 250µl/well of coating solution is added to each well; typically preparing 8 wells/plate. The coated plate is incubated at 37˚C for 1 hour, then the coating solution is removed and the wells washed with 1x PBS pH 7.4 once before plating the cells.

Thawing Cells: Cells are removed from liquid nitrogen and thawed in a 37˚C water bath for 2 minutes, until the cell solution is thawed. The 1mL solution is transferred, drop-wise, to 5mL of warmed maintenance media (see table 3.5) and the cryovial rinsed with 1mL media to collect residue cells. The cells are centrifuged at 300 *xg* for 3 minutes until a cell pellet is formed, then after discarding the freezing media, the pellet is re-suspended in 1mL of fresh media for cell counting (Appendix B).

Plating Density: Human neural stem cells are plated at a density of 225,000 cells/well on 24-well plates.

Maintenance: Cells are maintained in 500µl/well (24-well plates) of maintenance media, and a 50% media change is required every 2 days.

Passaging Cells: To passage cells, all media is removed from the wells and 250µl/well of Accutase (Sigma Aldrich # A6964) is added to dissociate and detach the adherent monolayer cells. Placing the plate in the incubator at 37˚C allows the cells to detach within 5 minutes. 250µl/well of media is added to cease dissociation and the cell solution is transferred to a centrifuge tube; centrifuged at 300 *xg* for 3-4 minutes to form a cell pellet which is subsequently re-suspended in 1mL of fresh media for cell counting. 10µl of cell solution is mixed with 10µl of trypan blue (1:1), and 10µl is transferred to a haemocytometer for the cell count (refer to Appendix B).

Freezing Cells: To freeze cells, the cells are collected as described above for the passaging method, and centrifuged and re-suspended in 1mL of freezing medium (see table 3.5). Cells are frozen at a density of $1x10^6$ cells/1mL/vial.

Solution	Components	Concentrations prep 40mL	
Geltrex Coating Solution	Geltrex (12mg/mL stock) Maintenance Media	8.6µg 1mL	332µl 40mL
Maintenance Media	Neuro-Sure Neural Stem Cell Medium #ASM-4011	1x	40mL
Freezing Media	Neuro-Crest Stem Cell Medium #ASM-4021 DMSO	80% 20%	32mL 8mL

Table 3.5: Solutions for Culturing Human Neural Stem Cells. *The maintenance media is produced and provided by Applied Stem Cell with no addition supplements added before use.*

3.2.5 ACH Commercial Cell Line

Supplier: ReproCell Lot #: A01LML20 and #A01NN13

Preparing Culture Surfaces: Plates are coated with 0.002% Poly-L-Orthinine (see table 3.6), 250µl/well on 24-well plates and incubated at 37˚C for 2 hours. The wells are then washed twice with 1x PBS pH 7.4 before adding the coating solution (see table 3.6) provided by supplier and incubated at 37˚C overnight.

Thawing Cells: The ReproCell kits provide a thawing medium, and a maintenance media (40mL) to which 720µL of 'Additive A' is added, referred to herein as maturation media (table 3.6). The thawing media was defrosted at 4˚C for 2 hours and then warmed in a 37˚C water bath before use. The cryovial was submerged for 90 seconds exactly, then transferred to 10mL of warmed thawing media. Cells were then centrifuged at 300 *xg* for 5 minutes and re-suspended in 1mL of maturation media for cell count using a haemocytometer (refer to Appendix B).

Plating Density: Cells were plated at 225,000cells/well on 24-well plates.

Maintenance: Cells were grown for 19 days before an experiment; as recommended by the supplier, and maintained in maturation media (table 3.6). 50% media changes were carried out every 3-5 days.

Passaging: This cell line is a differentiated neuronal population so does not proliferate or require passaging.

Solution	Components	Concentration (prep 40mL)
PLO Coating Solution	Poly-L-Orthinine 0.1%	0.002%
Coating Solution	Provided by Supplier- no dilution	
Thawing Media	Provided by Supplier- no dilution	
Maturation Media	Maintenance Media	40mL
	Additive 'A'	720µL
	Penicillin/Streptomycin	400µl

Table 3.6: Solutions for the Culturing of ACH ReproCell Cell Line. See Appendices for Suppliers and Product Codes.

3.2.6 DA Commercial Cell Line

Supplier: ReproCell Lot #: A01025 and #A01NN28

Preparing Culture Surfaces: Plates are coated with 0.002% Poly-L-Orthinine (see table 3.7), 250µl/well on 24-well plates and incubated at 37˚C for 2 hours. The wells are then washed twice with 1x PBS pH 7.4 before adding the coating solution (see table 14) provided by supplier and incubated at 37˚C overnight.

Thawing Cells: The ReproCell kits provide a thawing medium, and a maintenance media (40mL) to which 520µl of 'Additive A' is added as directed (maturation media, table 3.7). The thawing media was defrosted at 4˚C for 2 hours and then warmed in a 37˚C water bath before use. The cryovial was submerged for 90 seconds exactly; then transferred to 10mL of warmed thawing media. Cells were then centrifuged at 300 *xg* for 5 minutes and re-suspended in 1mL of maturation media for cell count using a haemocytometer (refer to Appendix B).

Plating Density: Cells were plated at 225,000cells/well on 24-well plates.

Maintenance: Cells were grown for 19 days before an experiment; as recommended by the supplier, and maintained in maturation media (table 3.7). 50% media changes were carried out every 3-5 days.

Passaging: This cell line is a differentiated neuronal population so does not proliferate or require passaging.

Solution	Components	Concentration (prep 40mL)
PLO Coating Solution	Poly-L-Orthinine	0.002%
Coating Solution	Provided by Supplier- no dilution	
Thawing Media	Provided by Supplier- no dilution	
Maturation Media	Supplied Media	40mL
	Additive 'A'	520µl
	Penicillin/Streptomycin	400µl

Table 3.7: Solutions for the Culturing of DA ReproCell Cell Line.

3.2.7 iCell Neurons

Supplier: Cellular Dynamics International

Preparing Culture Surfaces: Plates were coated in 0.1% Poly-L-Orthinine solution (250µl/well on 24-well plates) and incubated at 37˚C for 1 hour. The solution was removed and each well was washed three times in 1xPBS pH7.4. Matrigel coating solution was prepared (table 3.8) and added to each well (250µl/well) and incubated at 37˚C for 30 minutes. The matrigel solution was removed and with no need for washing the wells, the cells can be plated immediately. Plates can also be prepared and stored with maintenance media (see table 3.8) in the wells at 4˚C until required for use.

Thawing Cells: The maintenance media (table 3.8) was warmed to room temperature and the cryovial immersed in 37˚C water bath for exactly 3 minutes; Cellular Dynamics recommendation. In the safety cabinet, the contents of the cryovial were transferred to a 50mL centrifuge tube and rinsed with 1mL of media to collect residue cells. The media is added drop-wise, to minimise osmotic shock, to the cell suspension. 8mL of warmed media was added to the cells and centrifuged at 300 *xg* for 3 minutes. Cells were then re-suspended in 1mL of fresh maintenance media and counted using a haemocytometer (refer to Appendix B).

Plating Density: Cells were plated at 40,000 cells/well on 96 well plates (100µl media/well) and 225,000 cells/well on 24 well plates (500µl media/well).

Maintenance: A complete media change was carried out the day after plating the cells, and 50% media change (250µl or 50µl/well) 2-3 days thereafter.

Passaging: These cells are differentiated neurons so do not proliferate or require passaging. They are useable for experimental work 4-7 days after plating.

Solution	Components	Concentration
Maintenance Media	Supplied Media	97mL
	Supplement	2mL
	Penicillin/Streptomycin	1mL
Matrigel Solution	Matrigel stock solution	62.7µl
	Complete Maintenance Media	8mL

Table 3.8: Solutions for the Culturing of iCell Neurons. See Appendices for Suppliers and Product Codes.

3.3 Results- Culture and Growth of Cell Lines

This section shows the results from analysis of culture conditions and maintenance and growth of each of the selected cell lines. According to the principles of the criteria 1; ease of culture and growth of cell lines, the proliferation rate, culture maintenance protocol ease and repeatability of culture conditions were critically analysed. To proceed to criteria 2 analysis; expression of SNAP-25, the project defines the necessity for the cell line to be easy to culture, provide a maintenance protocol with minimal opportunity for variation and repeatedly grow well following cell thawing, and routine passaging and culture requirements.

3.3.1 Neuroblastoma Cell Lines

Cell counts at each passage for the selected neuroblastoma cell lines BE(2)-C, SH-SY5Y and IMR-32 provided an indication of the cell line proliferation rate or doubling time. Figure 3.7 below displays the comparative neuroblastoma cell lines and proliferation numbers at passages number 1-4 following initial thawing from three replicate cultures.

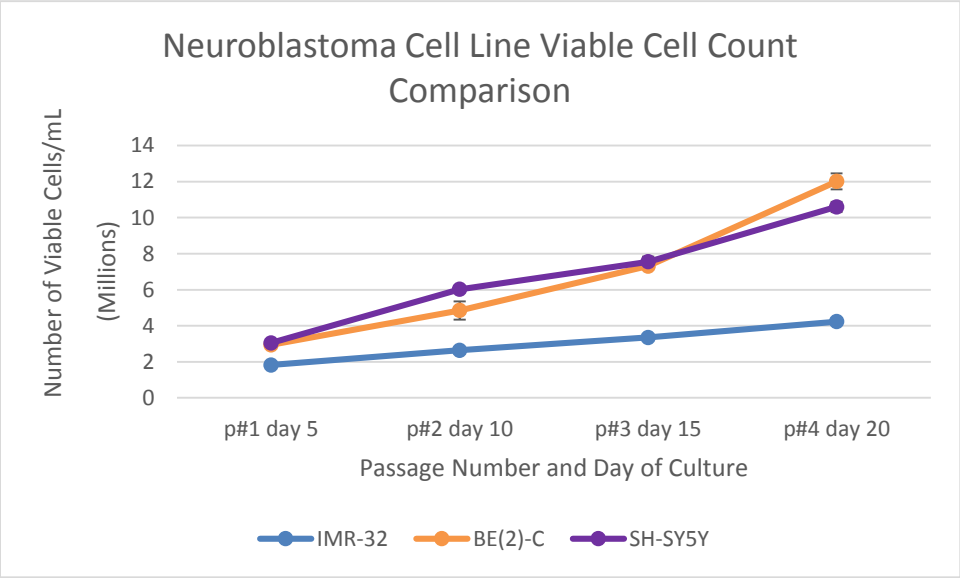


Figure 3.7: Neuroblastoma Cell Counts at Passage. *Comparative line graph demonstrating the proliferation of neuroblastoma cell lines IMR-32, BE(2)-C and SH-SY5Y through indicative viable cell count numbers at each passage. The graph shows three replicate cultures and the error bars represent the SEM. N=3 p=0.1699, showing no statistical difference between cell number at each passage in each of the three-replicate experiments (one-way ANOVA).*

The line graph in figure 3.7 above indicates the comparable proliferation rate of the neuroblastoma cell lines, showing cell number at four consecutive passages. The SH-SY5Y double in number approximately every 5 days providing abundant cells for sample collection. The BE(2)-C cells also proliferate well, with the number of cells duplicated between day 5 and 10 but the IMR-32 were slower. Given the issues with maintenance and plating of the cells previously described, the numbers of adhered and viable cells were reduced in contrast to the two other neuroblastoma cell lines. The IMR-32 took nearly 20 days to double in number; in comparison to the two other neuroblastoma cell lines this is a considerably longer period of proliferating cells to provide adequate cell number for future experiments.

3.3.1.1 BE(2)-C Cell Observations

Three defining principles for analysis of cell maintenance and growth were considered; proliferation, ease of culture maintenance and repeatability of culture conditions. The BE(2)-C neuroblastoma cell line proliferates well, doubling in number of cells between 5 and 10 days; providing an opportunity for ample stock for required experimental screening. The cells are easy to maintain; with no coating required before plating and the cells attach well to Corning flasks, within 12 hours. Media changes every 3-5 days, which for this project is defined as low maintenance. Plating on 25cm² flasks caused the requirement for cell passage to be frequent, every 4-5 days, which opens a risk of error or variables in culture conditions, but altering the culture flask size could reduce this potential issue by subsequently decreasing the frequency of passaging cells.

Principle	Ease
Proliferation	✓✓✓
Culture Maintenance	✓✓
Repeatability of Culture Conditions	✓✓✓

Table 3.9: Principles for Criteria 1 Applied to BE(2)-C Neuroblastoma Cell Line. *1-3 ticks associated with analysis of ease of each criteria for BE(2)-C cells. One tick to three ticks grading progressively indicate the level of ease; one is difficult to maintain or slow-growing and three ticks is very easy to maintain and high proliferation rate.*

The proliferation, ease of culture and maintenance of the cell line and the repeatability of culture conditions are graded in table 3.9 above. The culture maintenance was deemed slightly less desirable in comparison to the repeatability of culture and proliferation of the BE(2)-C cells, though still significantly better than some cell lines discussed in following results. This was due to the more complex make-up of the maintenance media and its potential to provide opportunity for risk in a routine working laboratory. The cells were

simple to culture, but there is a small risk that elements of the recipe for the complex media could be missed in preparation and a higher number of additional components to a basal media increases the risk of potential contamination. As culture maintenance is performed in aseptic conditions this risk should be minimal but worth noting when selecting a suitable cell line for a GMP-compliant assay. Three ticks were assigned with the evaluation of both proliferation and repeatability of culture conditions, signifying that the BE(2)-C cells proliferated well, and consistently.

It is assured by the supplier that after plating, the BE(2)-C cells adhere to the culture-ware growing in aggregates, demonstrating morphology with short neurite-like cell processes. The image captured from the cells plated for this project (figure 3.8a) recapitulates these cell attributes adhering to the expectations for cell morphology. It is expected that, following initiation of neuronal differentiation with a recommended protocol, as discussed in detail in the following chapter 4, dendrite extension is expected as the cells create neuronal network function. The BE(2)-C cells, in an undifferentiated state, show short neurite-like processes and the morphology and growth is as expected.

The captured image in figure 3.8a below indicates the morphology of the cells, which is comparable to the morphology shown in the image, figure 3.8b, provided by the supplier.

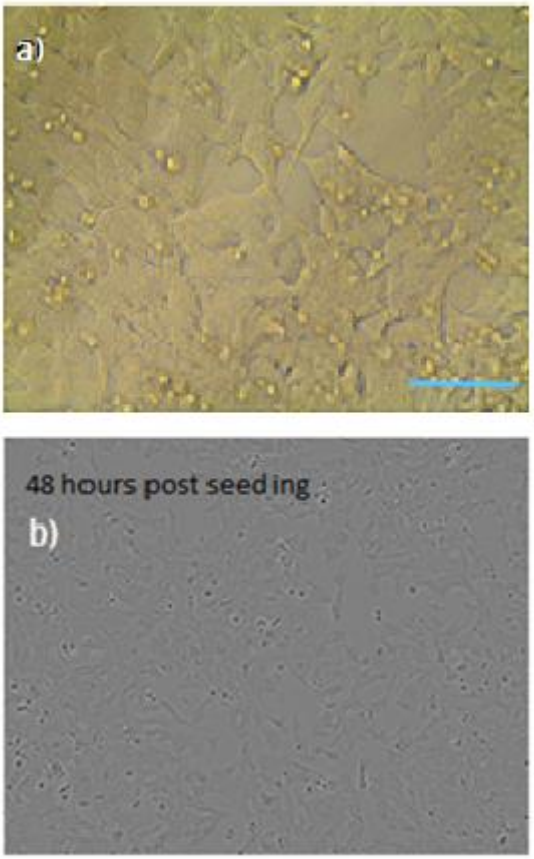


Figure 3.8: BE(2)-C Neuroblastoma Cell Line Phase Contrast Images. ***(a)*** *image of BE(2)-C cells captured 48-hours post-seeding. Passage#12 cells. Scale bar 10µm.* ***(b)*** *image previously shown in figure 3.1; images from supplier Public Health England 48-hours post-seeding.*

3.3.1.2 SH-SY5Y Cell Observations

Application of the three principles to the SH-SY5Y cell line indicates that this neuroblastoma cell line is comparable to that of the previously described (BE2)-C cells. The SH-SY5Y cells proliferate rapidly, doubling in cell number after approximately 5 days. Similarly, the cells do not require a pre-coating of culture-ware before plating and will adhere to the flask surface within 12 hours of plating or passaging. The cells detach well with the use of trypsin for passaging, and the maintenance protocol is minimal in relation to media changes; required every 3-5 days. As mentioned for the BE(2)-C cells, the rapid proliferation rate meant that plating the cells on 25cm² flasks significantly increased the requirement for and frequency of cell passage, again which can be minimised with the use of larger flasks.

Principle	Ease
Proliferation	✓✓✓
Culture Maintenance	✓✓
Repeatability of Culture Conditions	✓✓✓

Table 3.10: Principles for Criteria 1 Applied to SH-SY5Y Neuroblastoma Cell Line. *1-3 ticks associated with analysis of ease of each criteria for SH-SY5Y cells. One tick to three ticks progressively indicates the level of ease; one is difficult to maintain or slow-growing and three ticks is very easy to maintain and high proliferation rate.*

The proliferation, ease of culture and maintenance of the SH-SY5Y cell line and the repeatability of culture conditions are graded in table 3.10 above. The observations made of the SH-SY5Y cell line were similar to those of the BE(2)-C cells. Three ticks were assigned with the evaluation of both proliferation and repeatability of culture conditions, signifying that the SH-SY5Y cells proliferated well, and consistently. The cells were simple to culture, but the more complex make-up of the maintenance media would allow for potential issues to occur, i.e. elements of the recipe could be missed in preparation and a higher number of

additional components to a basal media increases the risk of potential contamination.

The supplier Public Health England states that the SH-SY5Y cells will grow as an adherent monolayer with short neurite-like processes. The image in figure 3.9a shows that the plated cells match the expected morphology, with similarities to the image in figure 13.9b from the supplier. Literature suggests that the length of neurites is extended with the application of differentiation protocols to the SH-SY5Y cells, enhancing dendrite branching and network formation (Teppola, Sarkanen et al. 2016). If synaptic function is determined as important for toxin sensitivity, a differentiation protocol will need to be applied to the SH-SY5Y to enhance sensitivity for use in a cell-based assay.

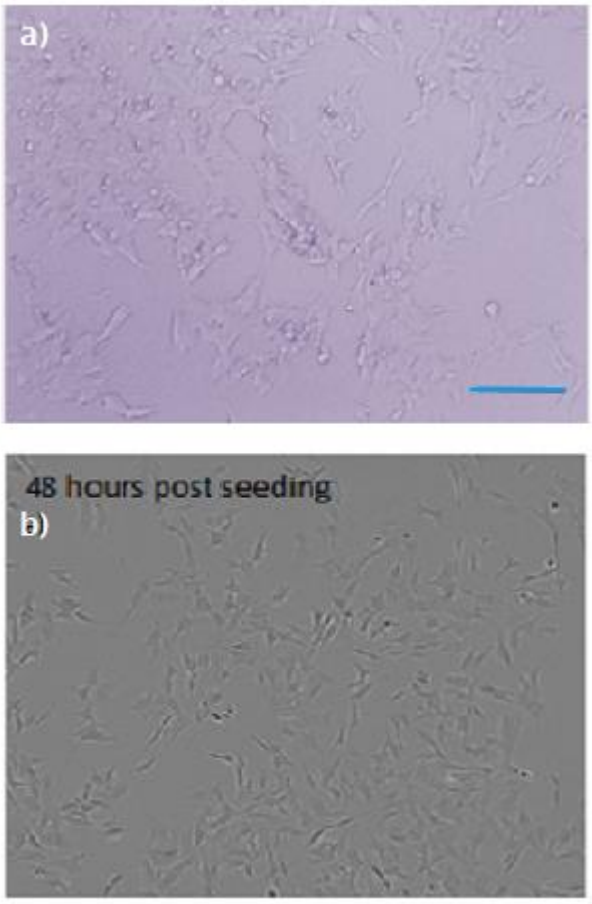


Figure 3.9: SH-SY5Y Neuroblastoma Cell Line Phase Contrast Images. *(a) image of SH-SY5Y cells captured 48-hours post-seeding. Passage#12 cells.* ***(b)*** *image previously shown in figure 3.2; images from supplier Public Health England 48-hours post-seeding. Scale bar 100µm.*

3.3.1.3 IMR-32 Cell Observations

The culture maintenance for the IMR-32 neuroblastoma cell line is simple; the media make-up contains less components and the passaging requirements and media changes are less frequent, minimising risk of variation, compared to the BE(2)-C and SH-SY5Y cell lines. The passaging of the IMR-32 cells is also easy, with the cells detaching easily without the use of trypsin summarising that the culture maintenance of these cells is appropriate and beneficial for the project requirements. Considering the proliferation of the cells, the doubling time is significantly longer than that of the BE(2)-C and SH-SY5Y cells, taking up to 20 days to double in number, with reference to figure 3.7.

Principle	Ease
Proliferation	✓
Culture Maintenance	✓✓✓
Repeatability of Culture Conditions	✓

Table 3.11: Principles for Criteria 1 Applied to IMR-32 Neuroblastoma Cell Line. *1-3 ticks associated with analysis of ease of each criteria for IMR-32 cells. One tick to three ticks progressively indicates the level of ease; one is difficult to maintain or slow-growing and three ticks is very easy to maintain and high proliferation rate.*

The proliferation capabilities of the IMR-32 cell line has been defined as low or insufficient, as the comparable proliferation rates of the neuroblastoma cell lines show that the time to amplify enough stock to replicate experiments would be significantly longer. Culture maintenance is summarised as a positive attribute of this cell line, with simple maintenance procedures, but the IMR-32 cells, much like the other neuroblastoma cell lines, will require a differentiation process to ensure sensitivity. The principle and requirement of repeatability of the culture conditions criteria was not met when analysing this culture, the issues with slow proliferation may be associated with the inability to maintain the cells in culture with the expected, suitable morphology.

Insurance of adherence of this apparent adherent cell line proved to be problematic. The supplier datasheet indicates that there is no requirement for a substrate to encourage cell adherence, but on Corning 25cm² flasks, as used for the BE(2)-C and SH-SY5Y cell lines, the IMR-32 would grow as suspended aggregates, with only a few cells, approximately 10% in each well, attaching to the plate. A slight movement of the plate causes the minimal number of adherent cells to detach and they do not re-attach when left in the incubator. A reduction in the concentration of FBS from 20% to 10% did not improve the adherence of the cells, and although altering the type of plate to NUNC 6 well plates (Thermo Scientific) encouraged a larger population of the cells to grow as an adherent monolayer, the cells continued to detach when the plate was moved (see figure 3.10a and 3.10b). The cells are supposed to be fibroblast-like adherent cells growing as a monolayer and appear as shown in figure 3.10c. Figure 3.10a shows cell in large aggregate formations and minimal adherent cells. Figure 3.10b shows improved adherence with the alteration of the culture surface, but still demonstrates a different morphology to that of the cells shown in figure 3.10c, from the supplier.

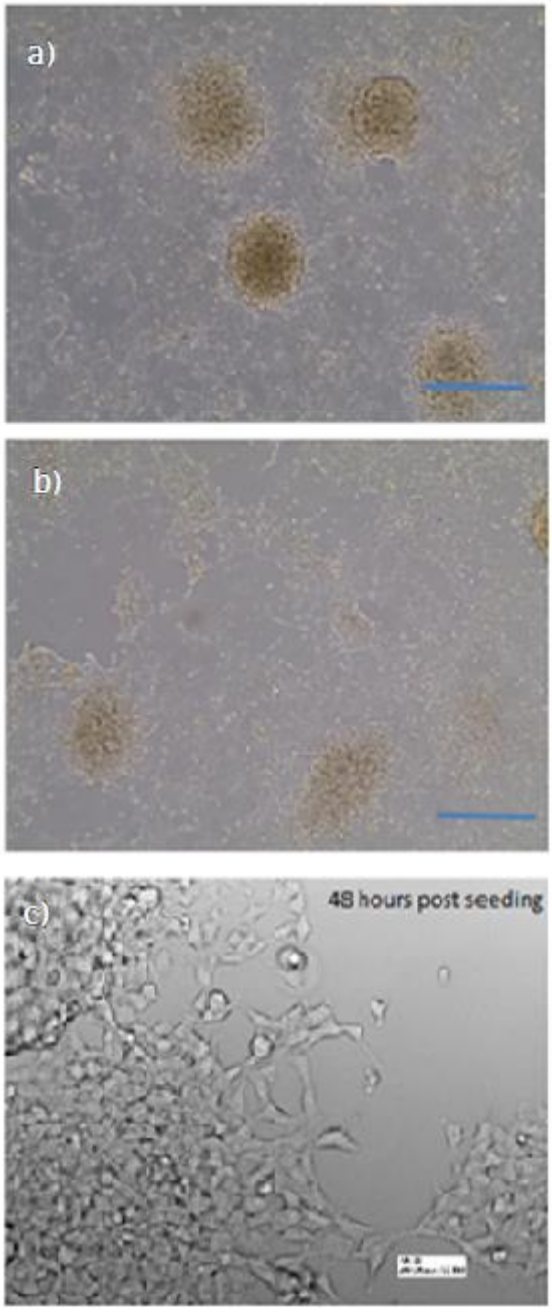


***Figure 3.10: IMR-32 Neuroblastoma Cell Line Phase Contrast Images**. **(a)** IMR-32 cells 48-hour post-seeding, Passage# 12, Corning Plates. **(b)** IMR-32 cells 48-hour post-seeding, Passage# 14, Nunc Plates, **(c)** image of IMR-32 cells, previously shown in figure 3.3, from supplier DMZ 48-hours post-seeding. Scale bar 100µm.*

3.3.1.4 Neuroblastoma Cell Lines Summary

To summarise, cell count data provided indication of the proliferation rate of each of the neuroblastoma cell lines, and observations of cell morphology ensured a reliable indication that the cells are growing efficiently, with expected morphology and characteristics. The SH-SY5Y and BE(2)-C cells proliferation rates indicate that abundant cell numbers for sample availability for future experiments could be quickly produced and that the cells could be available for use, undifferentiated, with a short start-up time in relation to use in a routine cell-based assay. The culture maintenance of the neuroblastoma cell lines is manageable and could be efficiently applied to a GMP-compliant laboratory experiment methodology, with the IMR-32 cells being simpler in frequency of passage requirements and media changes, therefore providing minimal risk of variation in media and maintenance procedures. It was documented by the cell line supplier that the SH-SY5Y cells are susceptible to high genetic variability coherent with higher passage numbers. This would suggest that a new stock vial will need to be purchased regularly, with frequent genetic expression evaluation to ensure repeatability of culture conditions if this cell line were to be selected for use in the cell-based assay. The IMR-32 are more problematic in their proliferation rate and repeatability of culture conditions. The cells are differing from the expected morphology, growing as aggregates, both suspended and adhered, rather than in a monolayer formation, and issues surrounding attachment may cause the cells to be challenging in use for experiments with differentiation and toxin treatments.

3.3.2 iPSC-derived Cell Lines

3.3.2.1 Human Neural Stem Cells Observations

Figure 3.11 below shows the cell morphology observed on plating of the hNSCs, captured 48 hours post-seeding. The images show that the density is sparse in comparison to the figure provided by the supplier.

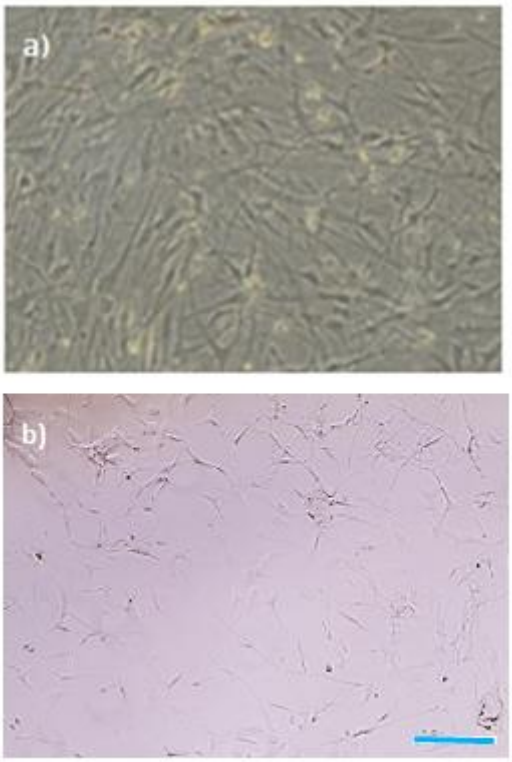


Figure 3.11: Applied Stem Cell Human Neural Stem Cells Phase Contrast Images. (a) *image previously shown in figure 3.4; images from supplier Applied Stem Cell 48-hours post-seeding.* ***(b)*** *image of hNSC cells captured 48-hours post-seeding. Passage#17 cells x20. Scale bar 100µm.*

The hNSCs were slow growing, in comparison with the other cell lines, taking up to 15 days to reach 95% confluence for passaging. It took approximately 60 days to produce enough cells for analysis of expression of SNAP-25.

The cell count graph below (figure 3.12) indicates the slow growth of the cells over a period of 51 days.

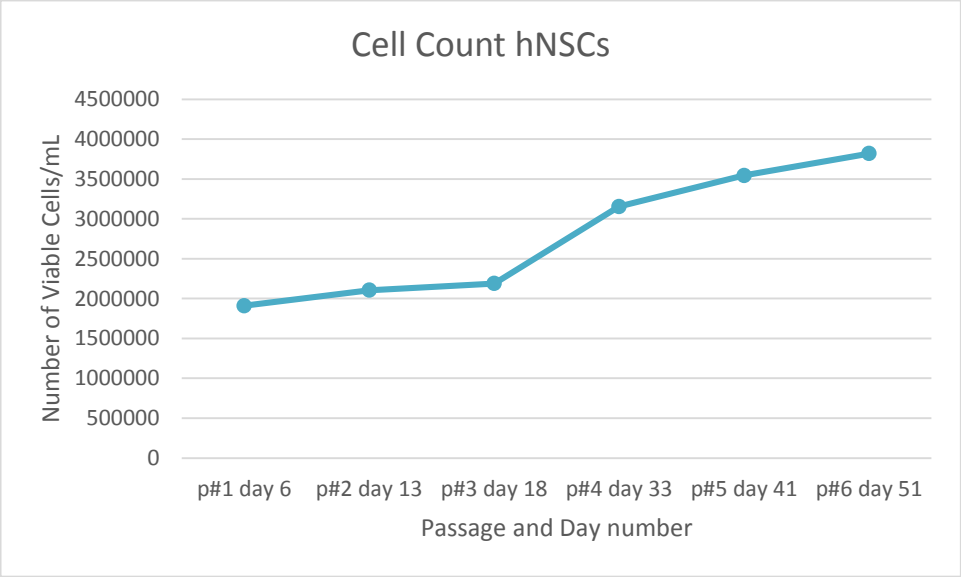


Figure 3.12: Cell Count of Human Neural Stem Cells. *The number of cells/mL are shown over a period of 51 days, data collected over six cell passages.*

The cells proliferate slowly over this time period, and over 51 days had not even doubled in number. This will limit the capabilities for this cell line for use in a cell-based assay as start-up time should be prompt and the samples should be abundant to provide several replicates and possible repeat testing experiments.

The protocol for the plating of the hNSCs is simple, with a one-step coating procedure using Geltrex but the cells require a media change every 2 days, which is more often than some of the other cell lines in comparison.

The matrix on which the cells are plated (Geltrex) provided reliable culture conditions and ensured cell adherence as recommended; but the slow growth and proliferation of these cells make them a challenge to use.

Principle	Ease
Proliferation	✓
Culture Maintenance	✓✓
Repeatability of Culture Conditions	✓

Table 3.12: Principles for Criteria 1 Applied to hNSCs Cell Line. *1-3 ticks associated with analysis of ease of each criteria for hNSCs cell line. One tick to three ticks progressively indicate the level of ease; one is difficult to maintain or slow-growing and three ticks is very easy to maintain and high proliferation rate.*

3.3.2.2 ReproCell ACH and ReproCell DA Cell Observations

Both the ACH and DA cell lines have been defined as low in relation to proliferation (table 3.13 below) given that they are a commercially differentiated cell line that do not proliferate; the cell number is final on initial plating. The cells are purchased as differentiated cholinergic (ACH) or dopaminergic (DA) neurons and the number of cells provided in one of the two vials for each cell line was a lot less than anticipated, (250000 cells/mL ACH and 305000 cells/mL DA cell line) limiting the number of cells available for the first set of experiments. The suggested cell density was 1 x 10^6 cells/mL for both the ACH and DA cell lines. The culture maintenance is simple and replicable as both cell lines are supplied with a commercial kit, containing coating solutions and supplements labelled as 'Additive A' to add to RPMI 1640 media for maturation media. It is a longer assigned time-frame for the cells to be ready for use; the coating of the plates incorporates an overnight incubation in provided coating solution following a 2-hour incubation of each well in Poly-L-Lysine. The maintenance of both the ACH and DA cell lines is simple, but the maturation time required is lengthy in that the recommended maintenance protocol provided with the cells states that the cells should be plated and maintained for 19 days before use for experiments or assays.

Principle	Ease ACH	Ease DA
Proliferation	✓	✓
Culture Maintenance	✓✓	✓✓
Repeatability of Culture Conditions	✓	✓

Table 3.13: Principles for Criteria 1 Applied to ReproCell ACH and DA Cell Lines. *1-3 ticks associated with analysis of ease of each criteria for ReproCell ACH and DA cells. One tick to three ticks progressively indicate the level of ease; one is difficult to maintain or slow-growing and three ticks is very easy to maintain and high proliferation rate.*

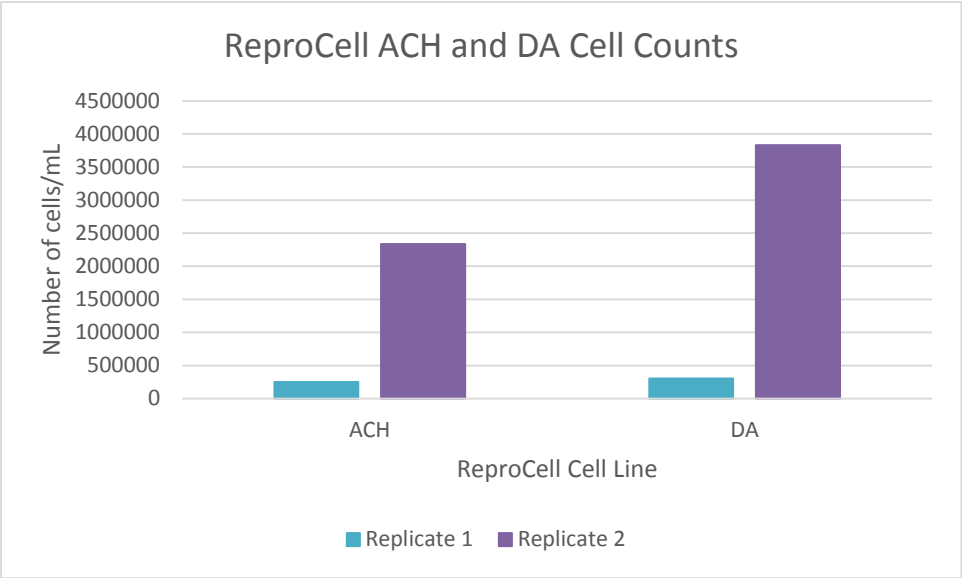


Figure 3.13: Bar Chart demonstrating ReproCell ACH and DA Cell Count Numbers at Initial Plating. *Cell count from plating densities of ACH and DA cell lines purchased from ReproCell.*

Although only two vials of each cell line (ACH and DA) were purchased, the cell density variability (figure 3.13) suggests an issue with selecting either the ACH or DA cell line for the use in a cell-based *in vitro* assay. The cell number needs to be consistent if the cell line does not allow for expansion with proliferation to provide the robustness and repeatability required. This justifies the confirmation that both the ACH and DA cell lines do not adhere to the principle of repeatability of culture conditions when focussing on cell density and availability irrespective of the robust and minimal variation capabilities of the culturing maintenance protocols.

3.3.2.3 iCell Neurons Cell Observations

The iCell neurons are purchased as differentiated GABAergic and glutamatergic neurons, so like to ReproCell ACH and DA cell lines, they do not proliferate. The cell line has subsequently only been graded as 'medium' in relation to proliferation (table 3.14). This reflects the improved consistency with cell number when compared to the densities of the ReproCell cell lines, and this consistency is portrayed consistently with each vial used in experiments.

The culture maintenance is simple and media and supplements are provided with the purchase of the cell line. The culture-ware coating procedure is more appealing, with regard to cell start-up time, than the ReproCell methodology, as there is no requirement for an overnight coating incubation. The cells grow well on the PLO and Matrigel coated plates, and this has been consistently shown in each experimental use of the cell line. The cells are easy to manage; with only half media changes, (250µl/well) required every 2-3 days. The cell density provided from each vial used was considerably higher than the ReproCell ACH and DA cell lines and consistent with each purchase (see figure 3.14).

Principle	Ease
Proliferation	✓
Culture Maintenance	✓✓✓
Repeatability of Culture Conditions	✓✓✓

Table 3.14: Principles for Criteria 1 Applied to iCell Neuron iPSC-derived Neurons. *1-3 ticks associated with analysis of ease of each criteria for iCell neurons. One tick to three ticks progressively indicate the level of ease; one is difficult to maintain or slow-growing and three ticks is very easy to maintain and high proliferation rate.*

The iCells were repeatedly successful in culture, adhering to the plate well, with low cell death and the culture conditions can therefore be perceived as repeatable; in relation to the cell number in each purchased vial and the success of culturing the cells. Although the cell line is purchased as differentiated neurons, which does not allow for cell proliferation, the cell density has been consistent and will provide enough viable cells for the intended experiments in the following chapters. The lack of proliferation can be identified as a negative element of this cell line, but only in relation to cost for use in a routine cell-based assay; for the minimising of risk of contamination and variability defined in the criteria for analysing culture conditions, the iCell neurons are a strong candidate in relation to ease of culture.

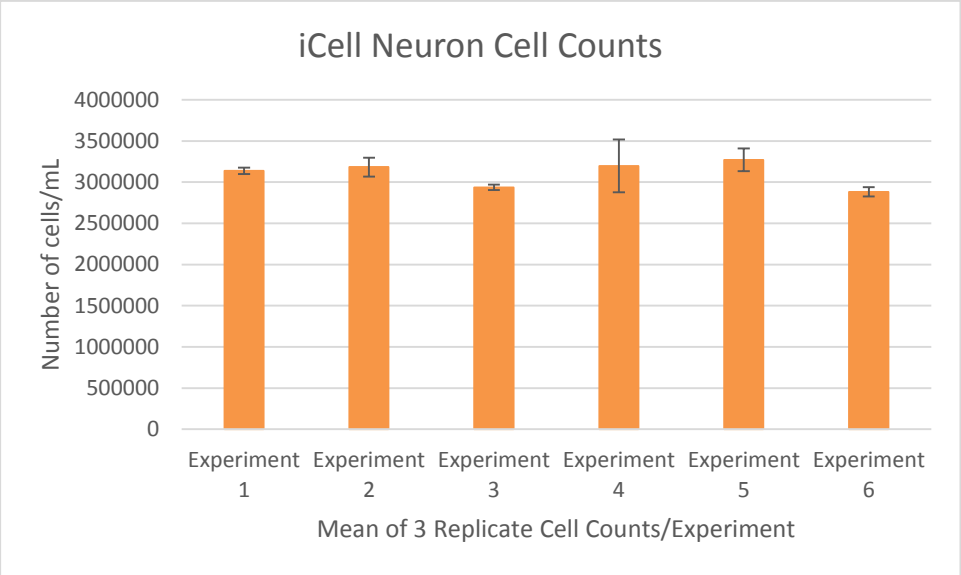


Figure 3.14: Bar Chart demonstrating iCell Neurons Cell Count Numbers at Initial Plating*. Graph demonstrating mean of 3 replicate cell counts, per experiment shown. (each experiment uses three vials of iCell neurons, with 6 independent experiment shown).*

3.3.2.4 iPSC-derived Cell Lines Summary

The selected iPSC-derived cell lines provided one passagable and proliferating candidate and two commercially differentiated cell lines, one of which has been used in previous published research for toxin assays; the iCell neurons. The human neural stem cells would provide an opportunity to differentiate the cells in-house, providing a more economically beneficial option, but this could provide opportunity for variability and error with reference to the growth and proliferation capabilities shown when using the cell line. The availability of cells 'for-use' will be significantly longer than the comparable ReproCell ACH, DA and iCell neurons cell lines set-up time. All three are easy to culture, with minimal maintenance required (for undifferentiated hNSCs from Applied stem Cell) and the medias and supplements required for cell line maturation are available from the supplier 'as-is' with minimal requirement for complicated media make up; resulting in possible variability.

3.4 Discussion – Culture and Growth of Cell Lines

3.4.1 Neuroblastoma Cell Lines

Analysing the growth of the neuroblastoma cell lines has indicated that the BE(2)-C and SH-SY5Y cell lines proliferate rapidly, providing abundant stock for use in experiments, which meets the defined criteria of ease of culture. The cells can be available for use promptly, if used undifferentiated, and double in cell numbers within 5 days. The media used for the BE(2)-C and SH-SY5Y cell lines is the same, and both use six reagents, including antibiotics if required, which is complex in comparison to some of the other cell lines.

Completing several replicates of freezing, thawing, proliferating, and passaging each of the cells lines has indicated the neuroblastoma cell lines repeatability and robustness of culture conditions. The BE(2)-C and SH-SY5Y cell lines consistently grow well in culture and provide a reliable number of viable cells with each passage, approximately doubling in number every 5-6 days. The cells adhere well to culture-ware, detach efficiently with the use of trypsin and adhere to plates within 12 hours. The continuation of analysis of the SH-SY5Y cell line is beneficial as it has been shown to demonstrate sensitivity to botulinum toxin (Purkiss, Friis et al. 2001) and could be a potential contender for use in the cell-based assay. The BE(2)-C cell line, as previously mentioned, was derived from the cell line SK-N-BE(2). A cell line named BE(2)-M17 was also isolated from SK-N-BE(2) and this cell line has been demonstrated to show sensitivity to toxin in literature (Lee, et al, 2008); so the comparable isolated BE(2)-C is an interesting option for continued analysis. The similar characteristics are the origin of the cell line, and high capacity to convert tyrosine to dopamine (Lee, et al, 2008).

In comparison, the remaining neuroblastoma cell line, IMR-32, however, is more problematic in culture. The cells proliferate more slowly than the comparable BE(2)-C and SH-SY5Y neuroblastoma cell lines, doubling in number in approximately 20 days, and the adherence of the cells is variable. Cells were initially plated on Corning 25cm² flasks, similarly to the other neuroblastoma cell lines, but the cells did not adhere after 12 hours. The flasks were observed, and images captured, at 48-hours and the adherence had not improved, with most of the cell population growing as suspended aggregates rather than the expected adherent monolayer (see figure 3.10).

Positively, the media composition for the IMR-32 cell line is simpler than the BE(2)-C and SH-SY5Y cells, using three reagents plus antibiotics if required, reducing the risk of error. However, the defined criteria of necessary repeatability of culture conditions cannot be applied to the IMR-32 cell line. Each passage was variable in relation to the number of cells adhering to the plate and the later the passage (higher passage number) the less cells attached to the plate.

To eliminate the use of this cell line at this stage of analysis of cell sensitivity would not be beneficial as the IMR-32 cell line demonstrates comparable characteristics to the neuroblastoma cell lines published in research for toxin sensitivity (Fernandez-Salas, Wang et al. 2012) (Lee, et al, 2008). Improvement in culturing ease was developed with the use of Nunc plates, which will allow for some experiments to be completed using this cell line.

Each of the neuroblastoma cell lines are analysed using the defined principles set out in the introduction to this chapter and demonstrated in the following table (table 3.15). BE(2)-C observations are shown in red, SH-SY5Y shown in purple and IMR-32 in orange.

Principle/Standard	Neuroblastoma Cell Line Observations	Action
Proliferation	Cells proliferate well, efficient doubling time and available cell number ✓ ✓ x	Consider cell line for criteria 2 analysis (expression of SNAP-25) **BE(2)-C, SH-SY5Y**
	Cells are slow growing, low cell number availability x x ✓	Eliminate cell line as a suitable candidate **IMR-32****
Culture Maintenance	Cell maintenance is simple; media changes fit in with a weekly work schedule, media make-up has minimal risk of variation ✓ ✓ ✓	Consider cell line for criteria 2 analysis (expression of SNAP-25) **BE(2)-C, SH-SY5Y, IMR-32**
	Maintenance is complex, frequent media changes required, complex media make-up which increases error risk x x x	Eliminate cell line as a suitable candidate
Repeatability of Culture conditions	Cell line consistently grows well, with expected morphology and is reliable to culture with minimal contaminations or errors ✓ ✓ x	Consider cell line for criteria 2 analysis (expression of SNAP-25) **BE(2)-C, SH-SY5Y**
	Cell culture is variable, high cell death, no adherence/differing morphology x x ✓	Eliminate cell line as a suitable candidate **IMR-32****

Table 3.15: Principles and Requirements for Consideration for Criteria 1; Ease of Culture and Maintenance. *The tabled criteria are considerations when analysing each of the selected cell lines for ease of culture and the standards for maintenance requirements. Observations for each of the neuroblastoma cell lines are shown in colour; BE(2)-C, SH-SY5Y, IMR-32. ** indicates suggestion of elimination based on the criteria.*

3.4.2 iPSC-derived Cell Lines

The human neural stem cells have been difficult to amplify; with the cells taking over 50 days to double in number after initial plating. Although the plating and maintenance protocol is simple in comparison to some of the other cell lines, with one simple coating procedure and a provided pre-made media for maturation of the cells, media changes are required every other day; more frequently than the other iPSC-derived cell lines comparatively. These cells do not adhere to the criteria of proliferation requirements, but in relation to the repeatability of culture conditions the cells grow and adhere well to the culture surface and the media make-up is simple in terms of maintaining the cell line. Although these cells are slow-growing consistently; several frozen stock from the initial stock vial demonstrated slow proliferation following thawing, the benefit of continuing analysis of this cell line is that the cells can be differentiated to be more sensitive for our purposes. This is a similar capability to that of the neuroblastoma cell lines, and in comparison to the commercially differentiated iCells and Reprocell cell lines, the hNSCs provide a cheaper cell culture option and subsequent economic benefit and enticement for a commercial cell-based assay.

The ReproCell ACH and DA cell lines are simple in relation to maintenance in that all reagents and the cell line are provided as a commercial kit. The pre-plating requirements are longer than other analysed cell lines, with an overnight incubation needed to coat the plates but as the cells are purchased differentiated as either cholinergic (ACH) or dopaminergic (DA) neurons the maintenance is simplified, eliminating the requirement for an extensive differentiation protocol. Although differentiation of the passageable cell lines has not yet been discussed, it is an important consideration in relation to ease of culture and growth and related time lines to provide utilisable cells for experimental testing. The number of cells on initial plating was variable, relating to the defined criteria of repeatable culture conditions, and as these cells do not proliferate, this could prove problematic in terms of reliable sample availability. Both the ACH and DA cell lines require a 19-day maturation in maintenance media before they can be used for testing, which may be comparable to required differentiation protocols for the hNSCs and neuroblastoma cell lines to improve sensitivity.

The iCells maintenance is reduced in comparison to the neuroblastoma cell lines and other commercial cell lines, with the supplier Cellular Dynamics indicating that the cells can be used 4 days after plating. Like the ReproCell commercial cell lines, the iCells do not proliferate, but the cell density provided from each vial used was considerably higher than the comparable commercially differentiated cell lines, and consistent with each purchase (see figure 3.14). The efficiency of this cell line is excellent in relation to use for a routine assay in a commercial laboratory, with quick set up before being able to use, easy maintenance and no need for passaging and risking contamination (minimal handling before use for testing). All three commercial cell lines are promising for the use in a cell-based assay, with high benefit in the minimal variability associated with differentiating cells in house.

Each cell line, at this stage, remains as a probable candidate for use in a cell-based assay for the potency testing of Botulinum toxin type-A based on the ease of culture, proliferation and repeatability of culture conditions. Each of the iPSC-derived cell lines are analysed using the defined principles set out in the introduction to this chapter and demonstrated in the following table (table 3.16). Applied Stem Cell hNSCs observations are shown in red, ReproCell ACH shown in purple, ReproCell DA shown in green and the iCell observations are shown in in orange.

Principle/Standard	iPSC-derived Cell Line Observations	Action
Proliferation	Cells proliferate well, efficient doubling time and available cell number x n/a n/a n/a	Consider cell line for criteria 2 analysis (expression of SNAP-25) ACH, DA, iCells
	Cells are slow growing, low cell number availability x x x ✓	Eliminate cell line as a suitable candidate hNSCs**
Culture Maintenance	Cell maintenance is simple; media changes fit in with a weekly work schedule, media make-up has minimal risk of variation ✓ ✓ ✓ ✓	Consider cell line for criteria 2 analysis (expression of SNAP-25) hNSCs, ACH, DA, iCells
	Maintenance is complex, frequent media changes required, complex media make-up which increases error risk x x x x	Eliminate cell line as a suitable candidate
Repeatability of Culture conditions	Cell line consistently grows well, with expected morphology and is reliable to culture with minimal contaminations or errors x ? ? ✓	Consider cell line for criteria 2 analysis (expression of SNAP-25) ACH, DA, iCells
	Cell culture is variable, high cell death, no adherence/differing morphology ✓ ? ? x	Eliminate cell line as a suitable candidate hNSCs**

Table 3.16: Principles and Requirements for Consideration for Criteria 1; Ease of Culture and Maintenance. *The tabled criteria are considerations when analysing each of the selected cell lines for ease of culture and the standards for maintenance requirements. Observations for each of the iPSC-derived cell lines are shown in colour; hNSCs, ACH, DA, iCells. ** indicates suggestion of elimination based on the criteria.*

3.5 Conclusion – Culture and Growth of Cell Lines

It is concluded, based on the assessment of ease of culture, proliferation and growth of each cell line in Chapter 3 that all of the cell lines will proceed to criteria 2, and will be screened for SNAP-25 expression. Several considerations require attention when analysing SNAP-25 expression of each of the cell lines. This refers to the variable density of the ReproCell ACH and DA cell lines, the slow proliferation of the human neural stem cells and the issues concerning adherence concerning the IMR-32 neuroblastoma cell line. Table 3.17 below summarises the ease of culture and growth for each of the cell lines and the cell lines considered for further analysis for suitability. The culture and growth of each cell line are graded specifically on the ease of each aspect. The BE(2)-C, SH-SY5Y and iCell neurons are highlighted as the most successful candidates at this stage with relation to ease of culture, maintenance and growth.

Cell Line	hNSCs	BE(2)-C	SH-SY5Y	IMR-32	ACH	DA	iCells
Proliferation	✓✓	✓✓✓	✓✓✓	✓	✓	✓	✓
Culture Maintenance	✓✓	✓✓	✓✓	✓✓✓	✓✓	✓✓	✓✓✓
Repeatability of Culture Conditions	✓	✓✓✓	✓✓✓	✓	✓	✓	✓✓✓
Further Analysis?	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**

Table 3.17: Cell Line Conclusions Following Analysis of Ease of Culture and Growth. *Table outlines conclusive cell line observations and the selection of the cell lines for analysis of SNAP-25 expression in Chapter 4. One tick to three ticks progressively indicate the level of ease; one is difficult to maintain or slow-growing and three ticks is very easy to maintain and high proliferation rate.*

Chapter 4: Analysis of SNAP-25 Expression

4.1 Introduction

Analysis of the ease of culture and growth of the seven investigated cell lines, defined in Chapter 3, suggests that, at this stage in the screening process, each candidate remains as a potential sensitive option for use in a cell-based assay for testing of BoNT-A. Proliferation, ease of culture maintenance and repeatability of culture conditions was assessed for each cell line, and conclusions from this analysis highlights that at this stage, BE(2)-C, SH-SY5Y and the iCell neurons are the most favourable cell lines. The hNSCs, ReproCell ACH and DA cell lines are more variable in the repeatability of culture conditions, with varying cell densities and slow proliferation, and the IMR-32 could prove problematic for further analysis concerning adherence and expected morphology. It is defined as an essential element to assay design that the cell line is easy to manage, so these conclusions regarding the results from criteria one will be considered with the results of the analysis of SNAP-25 expression for each cell line (criteria 2).

As described in the project plan in Chapter 2, the cell lines ability to express SNAP-25 is of upmost importance when designing a BoNT-A specific assay, as SNAP-25 is the SNARE target of toxin activity. The following chapter considers the requirements of criteria 2 in the approach to selecting a cell line for the testing of Botulinum Toxin; analysis of SNAP-25 expression. SNAP-25 forms part of the SNARE core complex that mediates synaptic vesicle fusion with the plasma membrane, enabling the release of neurotransmitters, specifically acetylcholine. SNAP-25 specifically is targeted by Botulinum Toxin Type-A, and is therefore a compulsory component in a cell-based assay. The cell line selected must express abundant, or at least detectable, SNAP-25 for the assay to be a success, allowing the detection of toxin activity based on its effect on the SNAP-25 protein. Therefore, the next consideration in the approach to selecting a sensitive cell line is to ensure that SNAP-25 is expressed to a detectable limit, and is repeatedly expressed at comparable levels in replicate experiments, for robustness and reliability. Expression of SNAP-25 protein is an essential component of cell line suitability for use in the testing of Botulinum toxin type-

A, and therefore cells that are unreliable in the expression of SNAP-25 will be eliminated.

There are principles and standards, defined in table 4.1 below, that should be adhered to in the determination of proceeding with a cell line to the analysis of toxin sensitivity (criteria 3 defined in the project plan) following analysis SNAP-25 expression. These standards are important considerations for the overall suitability of a cell line for this project.

Principle/Standard	Cell Line Observations	Action
SNAP-25 Expression	SNAP-25 is expressed and detectable	Consider cell line for criteria 3 analysis (toxin sensitivity)
	SNAP-25 cannot be detected following repeated experiments	Eliminate cell line as a suitable candidate
Repeatability of SNAP-25 Expression	SNAP-25 is consistently expressed and detectable in repeated experiments	Consider cell line for criteria 3 analysis (toxin sensitivity)
	SNAP-25 expression and detection is inconsistent in repeated experiments	Eliminate cell line as a suitable candidate
Differentiation Protocols and Requirements **Neuroblastoma Cell Lines; BE(2)-C, SH-SY5Y & IMR-32** **iPSC-derived Cell Line; Human neural stem cells**	Cells express SNAP-25 without differentiation requirements	Consider cell line for criteria 3 analysis (toxin sensitivity)
	Cells differentiate easily with a standardised procedure, and subsequent SNAP-25 expression is improved	
	Cells do not respond to differentiation protocol	Eliminate cell line as a suitable candidate
	Cells require a complex, variable protocol with a mixed population of cells (that cannot be consistently replicated)	

Table 4.1: Principles and Requirements for Consideration for Criteria 2; Expression of SNAP-25. *The tabled principles are considerations when analysing each of the selected cell lines for expression of SNAP-25 and the standards and requirements of the differentiation protocols.*

4.1.1 Defining Criteria in Analysing Expression of SNAP-25

The successful detection of SNAP-25 is an essential element in the determination of a cell lines potential sensitivity to Botulinum toxin type-A. The principles defined in table 4.1 above are approached in the table order; analysing SNAP-25 expression primarily, then assessing its repeatability in expression and detection, followed by the analysis of possible improvement of SNAP-25 expression following neuronal differentiation. The latter is focussed on the neuroblastoma cell lines and the human neural stem cell line selected for screening, as the iPSC-derived cell lines are differentiated neurons when purchased. SNAP-25 expression is analysed primarily using the Western Blot methodology and progressively analysed using mass spectrometry. This detection method has been introduced at this stage in the project with the intention of screening mass spectrometry as a potential detection method for use in the cell-based assay. SNAP-25 detection is essential, and the capabilities of mass spectrometry can be assessed when analysing the expression of SNAP-25.

The second stage of cell screening, which also provides optimisation opportunity of the mass spectrometry methodology and its potential, is to assess the reproducibility of SNAP-25 expression or detection. Repeated experiments provide the data to assess the cell lines capability of robust expression and the vigour of both of the utilisable detection methods Western Blot and mass spectrometry.

The neuroblastoma cell lines BE(2)-C, SH-SY5Y and IMR-32 are undifferentiated cell lines, and the SNAP-25 expression is analysed in both undifferentiated and differentiated cell samples to assess improved expression as a result of neuronal differentiation. Exposing the cell lines to differentiating reagents could improve the cells capability of expressing SNAP-25; aiming to differentiate the cells into neurons proposed to be most sensitive to botulinum toxin type-A.

4.1.2 Neuronal Differentiation of Neuroblastoma Cell Lines

Differentiation can be simply defined as the process that changes a cell from one type to another, more specialised, cell type. The differentiation process occurs throughout neural development, and the level of cell potency defines the cells ability to differentiate into other cell types. Pluripotent cells have the capability to differentiate into a range of cell types, multipotent cells differentiate into closely related cell types and unipotent cells are capable of self-renewal (Bear, et al, 2016). The figure below outlines the differentiation process from neural stem cell to specialised cells.

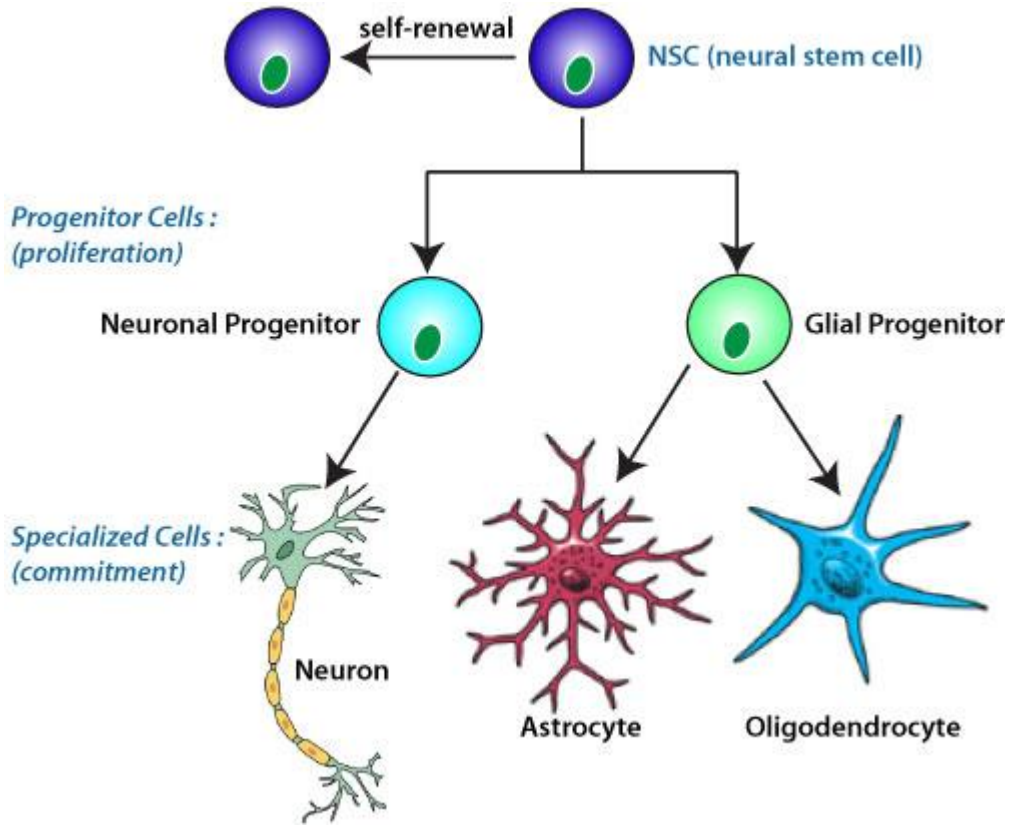


Figure 4.1: Indication of Neuronal Differentiation. *Image taken from System Biosciences Inc, 'Track Neural Differentiation Using qPCR' [online], California, 2017, 20/04/2017, https://www.systembio.com/stem-cell-research-neural-qpcr-profiler.*

Pluripotent stem cell differentiation approaches can allow for the differentiation into three primary germ layers; ectoderm, mesoderm and endoderm, within which the cells differentiate into multipotent stem cells and progenitor cells. Neural differentiation occurs from cells from the ectoderm layer, generating neural stem cells, neural progenitor cells and differentiated neurons. Differentiation of a neural precursor cell into a neuron begins with the appearance of neurites extending from the cell body; an axon and several dendrites depending on neuronal cell type.

The selected neuroblastoma cell lines and the human neural stem cell (hNSCs) cell line can be differentiated into cell types more sensitive to Botulinum toxin type-A, such as cholinergic, adrenergic, GABAergic or glutamatergic neurons. Sensitivity to toxin for these cell types has been documented in the use of SiMa cells (Fernandez-Salas, Wang et al. 2012) and iPSC-derived neuron cell lines (Whitemarsh, Strathman et al. 2012). Differentiation protocols allow for variability in cell populations, which will need to be monitored for a GMP-compliant assay; with the intent of minimising cell type variation for repeatability and consistency in assay protocol and results. If a cell line requires a differentiation protocol to ensure cell sensitivity to toxin it must be repeatable, reliable and the length of the protocol should be taken into consideration. A defining criterion is that the cell line is available for use for testing efficiently and promptly, thus a cell line that requires months to ensure a consistent differentiated neuron population would not be suitable for requirement.

The use of vasoactive intestinal peptide (VIP) was selected to screen as a potential differentiation protocol for the neuroblastoma cell lines. This differentiation method directs the cells into the neuronal lineage, potentially increasing SNAP-25 expression, which in turn could lead to improved toxin sensitivity. Literature suggests that VIP has been used to induce differentiation; with reference to dendritic cells (Chorny, Gonzalez-Rey et al. 2006) and human neuroblastoma cell lines (Pence and Shorter 1990). The neuroblastoma results in this study demonstrated cell proliferation suppression and morphological differentiation defined as neurite extension and cellular enlargement. Although cellular enlargement may not be a well-known factor of neuronal differentiation, there are a few studies that confirm this observation (Lloyd 2013) (Wallace and Das, 1983). The addition of growth factors and adaptations to the culture

conditions may be necessary to stimulate neuronal differentiation, but VIP should induce the early stages of differentiation.

Alongside the use of VIP induced-differentiation, a retinoic acid (RA) protocol was trialled on the neuroblastoma cell lines. The use of RA, as a neuronal differentiation agent, is well documented (Andrews 1984, Tonge and Andrews 2010), and is extensively used in cell culture neuronal differentiation protocols. Retinoic acid plays an important role in neural development; in axon regeneration and neuronal differentiation. RA-mediated differentiation occurs by the activation of transcription factor genes and cell signalling molecules, suggesting neuronal differentiation is regulated by complex molecular pathways (Dhara and Stice 2008). RA has been proven to promote neuronal differentiation in neural stem cells and induce functional maturation of differentiated neurons (Tan, Wang et al. 2015) and to induce the growth of cellular dendrites, typical of neuron structure. Results published by Tan, et al 2015, indicated that neural stem cells, when exposed to retinoic acid demonstrated a promoted cellular dendrite growth.

4.1.3 Analysis of Neuronal Differentiation- Immunocytochemistry

Analysis of the efficiency of the two selected differentiation protocols assesses the success of cellular neuronal differentiation. The morphology of the cells is expected to alter; the neuroblastoma cell lines already demonstrate neurite extension in relation to their neuron-like morphology when undifferentiated, but the cells should show reduced proliferation and extended neurite growth as a result of differentiation towards neuronal lineage. The aim of differentiating the neuroblastoma cell lines is to differentiate the cells into potentially sensitive cell types to increase the cells lines sensitivity to toxin by increasing the expression of SNAP-25. Western Blot analysis is used to assess the increase of SNAP-25 expression as a result of differentiation with RA. Detecting specific markers for differentiation and related neuronal cell types provides a quantitative analysis of differentiation. For analysis of the neuroblastoma cell lines, immunocytochemistry, using the markers Beta-III-Tubulin, ChAT and MNR2 was selected as the optimum analysis. The cell lines are analysed for the expression

of these markers at the same time point to assess the success of the VIP differentiation protocol.

Beta-III-Tubulin is an early neuronal cell marker in human nervous system (Katsetos, Legido et al. 2003) and detection of beta-III-tubulin with antibodies is a commonly used approach for confirmation of neuronal cell identity after differentiation induction of neural progenitors (Katsetos, Herman et al. 2003). Fluorescently detecting the presence of beta-III-tubulin will indicate the ability of the differentiation protocols to induce neuronal cell differentiation.

Acetylcholine is the primary neurotransmitter at the neuromuscular junction and is synthesised by all motor neurons in the spinal cord and brain stem (Bear, et al, 2016). Choline acetyltransferase (ChAT) is an enzyme that catalyses acetyl-coenzymeA to choline, to subsequently form the neurotransmitter acetylcholine (Govindasamy, Pedersen et al. 2004). The synthesis of acetylcholine involves this enzyme, which is only expressed in cholinergic neurons. ChAT is a prominent marker in cholinergic neurons in the central and peripheral nervous systems and is the most specific indicator for monitoring cholinergic systems in the functioning brain (Oda 1999). This marker was selected to assess the abundance of cholinergic neurons as a result of exposure to the VIP differentiation protocol. Botulinum toxin targets the neuromuscular junction, and cleaves SNARE proteins to prevent the release of acetylcholine thus it was deemed interesting to assess the sensitivity of cholinergic neurons to toxin for use in the cell-based assay. If cholinergic neurons are responsible for the production of acetylcholine, it is suggested that the cells are present at the neuromuscular junction and susceptible to interaction with Botulinum toxins *in vivo*.

It is well researched that SHH-signalling (Sonic hedgehog) is required for the generation of somatic motor neurons (Ericson, Muhr et al. 1995, Ericson, Morton et al. 1996) and it is suggested that SHH-triggered differentiation of neural progenitor cells into somatic motor neurons may be directed by the expression of MNR2 (Tanabe, William et al. 1998). Expression of MNR2 activates differentiation, characterised by the expression of ChAT and analysing the expression levels of MNR2 would indicate the cholinergic differentiation stage of the cultured neurons as a result of differentiation with VIP.

4.1.4 Introduction of an Alternative Detection Method- Mass Spectrometry

Mass Spectrometry is an analytical technique that identifies proteins based on mass analysis (Pitt 2009). The concepts and methodology are detailed in Chapter 7; a brief summary is that mass spectrometry was selected as a potential detection method in a botulinum toxin cell-based assay as it is a reliable and robust method. Electrospray ionisation allows a liquid sample to form a spray of charged droplets that are analysed in the mass spectrometer based on molecular mass and ion intensity. It is implied that detection of peptides can be performed using smaller sample volume than that used for Western Blot. The potential of mass spectrometry will be analysed, with the detection of SNAP-25 being the primary approach to optimising and assessing the method capabilities. Detecting whole SNAP-25 is indicative of assessing toxin activity with mass spectrometry, based on the future requirement of the detection and distinction between cleaved and uncleaved SNAP-25.

4.1.5 Assessing Growth Time- iCell Neurons

It is the supplier recommendation that the differentiated iCell neurons can be used between 4 and 21 days after initial plating. A set criteria in Chapter 3 is that a cell line can be available for use in routine testing of botulinum toxin in a short period of time, and are easy to maintain. The iCell neurons fit this criteria in that maintenance is simple, and the cells can be used within 4 days. To relate the cell growth time to the expression of SNAP-25 two time-points were selected to analyse the potential difference in expression of SNAP-25 which would suggest the ideal stage of growth that the iCells can be used for optimum SNAP-25 SNARE target expression. Cell samples were collected at 7 and 14 days to compare expression.

To summarise, to proceed to toxin sensitivity analysis a cell line should repeatedly express SNAP-25, demonstrated with consistent detection and expression with repeated experiments. Each cell line was assessed using the criteria defined in table 4.1 shown at the beginning of Chapter 4 and either discarded for suitability for use in a cell-based assay or selected to proceed to criteria 3; toxin sensitivity.

4.2 Methods – Analysis of SNAP-25 Expression

4.2.1 Neuronal Differentiation of Neuroblastoma Cell Lines

The VIP (Abcam #ab120180) was reconstituted to a concentrated stock solution of 50µM in 20mM TRIS and glycerol. For differentiating treatment on the cells, the stock was diluted to 1µM/well (24 well plates). BE(2)-C, SHSY5Y and IMR-32 cells were plated at densities according to sections 3.2.1, 3.2.2 and 3.2.3 (30,000cells/cm² and 10,000 cells/cm² respectively) and the cells were incubated in the VIP differentiation media (table 4.2), 500µl/well for BE(2)-C and SH-SY5Y 24-well plates, and 2mL for IMR-32 6-well plates. The cells were differentiated for 9 days, with media changes every 3 days. For the control sample the cells were incubated with TRIS glycerol control buffer, (table 4.2 below), with media also changed every 3 days.

Retinoic acid (RA) stock solution (50µM) (Sigma #R2625) was diluted in 500µl of DMSO and distributed onto the cells (BE(2)-C, SH-SY5Y and IMR-32) at 1µM per well. The cells differentiated for 9 days with RA in 24 well plates, with media changes completed on day 3 and 6. Cells were incubated with DMSO (diluent for RA) as the control, added at the same volume as the RA.

Solution	Components	Concentration/Volume
TRIS Glycerol Stock (Diluent of VIP stock)	TRIS hydrochloride 50µM Glycerol	750µl 750µl
VIP Differentiation Media	Maintenance Media Vasoactive intestinal peptide 100µM stock	495µl 1µM - 5µl
RA Differentiation Media	Maintenance Media Retinoic Acid 50µM stock	490µl 1µM - 10µl
Glycerol Buffer Control Media	Maintenance Media TRIS Glycerol Stock	495µl 5µl
DMSO Control Buffer	Maintenance media DMSO	490µl 10µl

Table 4.2: Neuroblastoma Differentiation Medias

4.2.2 Immunocytochemistry of VIP Incubated Cells

4.2.2.1 Fixing Cells

VIP-differentiated cells were fixed after 9 days, for immunocytochemistry, to analyse the presence of neuronal markers to determine the efficiency of the differentiation protocol. Culture media was removed from each well and each well was washed with 1x PBS pH7.4 three times. The cells were then fixed with 300µl/well 4% paraformaldehyde (PFA) for 20 minutes at room temperature. The PFA was removed and the wash steps repeated and, leaving 1mL of 1xPBS in each well, the plates were stored at 4◦C before staining.

4.2.2.2 Staining Cells

The PBS was removed from the wells and 300µl/well PBS 0.2% TritonX-100 (table 4.3) was added to each well to permeabilise the cells. This was incubated for 20 minutes at room temperature. The cells were then saturated in PBS 10% donkey serum 0.2% TritonX-100 (300µl/well) for 1 hour at 37˚C. Antibodies Beta-III-tubulin (1:500), MNR2 (1:5 and undiluted) and ChAT (1:50) were diluted in PBS 10% donkey serum 0.2% TritonX-100 (see table 4.3) and incubated with the fixed cells overnight at 4˚C.Following primary antibody incubation, the solution was removed from the wells, and each was washed three times with PBS. Secondary fluorescent antibodies were diluted in PBS 10% donkey serum (DS) 0.2% TritonX-100 at a concentration of 1:200 (see 4.4 below for different antibodies used) and added to the wells for 2 hours, and incubated at 37˚C. The secondary antibody solution was removed and replaced with DAPI solution 300µl/well and the plate was covered in foil to protect the cells from light. Before imaging, the wells were washed twice with PBS.

Solution	Components	Concentration
PBS 0.2% TritonX-100	Phosphate Buffered Saline TritonX-100	50mL 100µl
PBS 10% Donkey Serum 0.2% TritonX-100	Donkey Serum (DS) PBS 0.2% TritonX-100	400µl 3.6mL

Table 4.3: Solutions for Staining Cells

Antibody (species)	Supplier	Concentration
Anti-Beta-III-Tubulin (mouse)	Covance #MMS-435P	1:500
ChAT antibody (goat)	Millipore #AB144P	1:50
MNR2 (mouse)	DSHB #81.5C10	Undiluted or 1:5
Anti-mouse IgG (H+L) Alexa Fluor 488 (rabbit)	ThermoFisher #A27022	1:200
Anti-goat IgG (H+L) Alexa Fluor 568 (rabbit)	ThermoFisher #A27011	1:200
All antibodies for immunostaining were diluted in PBS 10% Donkey Serum 0.2% TritonX-100 as described in table 4.3 above.		

Table 4.4: Antibodies and Concentrations used in Immunostaining

The MNR2 antibody came with the supplier recommendation of using it at a concentration of 1:50, a concentration also confirmed by published research indicative of directed differentiation of neural stem cells and subsequent immunofluorescent staining of MNR2 positive cells (Hu and Zhang 2010). However, experiments using this concentration did not provide any results (results not shown) so it was decided to increase the concentration to 1:5 for the following experiments.

4.2.2.3 Imaging protocol

The cells stained with fluorescently tagged antibodies and DAPI solution as described previously, were observed under a fluorescent microscope (Leica DMIL inverted fluorescent microscope in the department of Clinical and Experimental Sciences, University of Southampton). Images were captured in each well and the clearest are shown in the results section 4.3.1. The same field of view was

imaged with blue, red and green filters according to the fluorescent emission, which is antibody dependant.

4.2.3 Sample Preparation for Western Blot Analysis of Neuronal Differentiation

The three neuroblastoma cell lines BE(2)-C, SH-SY5Y and IMR-32 were plated according to the densities in tables 4.5, 4.6 and 4.7 shown below and maintained in culture for 7 days. The issues with the adherence of the IMR-32 cells still applied in these experiments, which prevented immunocytochemistry analysis of the success of the differentiation protocols for this cell line. Each cell line was incubated in RA for 9 days, with media changes on days 3 and 6, and collected as per the cell collection methodology previously described. The samples were collected in RIPA buffer with protease inhibitors (see table 4.11 for composition) and stored at -80˚C until analysis. Three samples were collected of each of the neuroblastoma cell lines.

4.2.4 Undifferentiated Neuroblastoma Cell Line Sample Preparation for Western Blot and Mass Spectrometry

4.2.4.1 BE(2)-C

4.2.4.1.1 Western Blot

Samples were prepared for analysis of expression of SNAP-25, plating BE(2)-C cells, on 25cm² flasks and maintaining in maintenance media (table 4.5) for 7 days before using the cells in experimental conditions. These 7 days included a media change and the requirement for passage. Cells were passaged using trypsin to detach the cells from the flask. The passaging process was carried out by removing the media, adding 3mL/flask of trypsin and incubating at 37C for approximately 3 minutes. Adding 3mL of maintenance media ceases the cell dissociation process and the cells were centrifuged, and re-plated. Following the 7 days maturation before preparing cell samples, for consistency throughout the

experiments, the cells were passaged and plated at a density of 225.000 cells/well onto 24-well plates. The BE(2)-C cells did not respond well to such high density and the wells were 'overcrowded' causing cell detachment and less viable cells. The cells were re-plated according to the methodology in Chapter 3, plating 60,000 cells/well on 24-well plates and collecting comparable cell numbers in each sample, see table 4.5. Cells were collected in RIPA buffer using the methodology described in the following section 4.2.6.

4.2.4.1.2 Mass Spectrometry

High density cell samples were collected to both analyse the presence of SNAP-25 in the BE(2)-C cells and optimise the methodology of mass spectrometry. The undifferentiated BE(2)-C cells were plated and proliferated to collect a cell sample with approximately 5 x 10^6 cells for analysis. It is the intention to provide a high protein concentration to be able to specifically detect the peptides of SNAP-25 with the mass spectrometer. The cells were collected in triethylammonium bicarbonate (TEAB), following the methodology in section 4.2.6 below, and stored at -80˚C before analysis. The method of analysis using mass spectrometry is documented in Chapter 7, which documents the approach to the trialling of detection methods.

BE(2)-C Neuroblastoma Cell Line				
Method	Plating Density	Sample Collection	Maintenance Media 40mL	
Western Blot	30,000 cells/cm² 60,000 cells/well on 24-well plates 500µl media	10 wells in 60µl of RIPA buffer per sample	EMEM (EBSS) Hams F12 Non-essential amino acids Glutamine Heat inactivated Foetal Bovine Serum (FBS) Penicillin/Streptomycin	16.6mL 16.6mL 400µl 400µl 5.6mL 400µl
Mass Spectrometry	30,000 cells/cm² 750,000 cells/ 25cm² flask 10mL media	6 flasks in 100µl of TEAB buffer		

Table 4.5: BE(2)-C Neuroblastoma Cell Line Plating Density and Maintenance Media Required for SNAP-25 Expression Analysis Experiments. See Appendices for Suppliers and Product Codes.

4.2.4.2 SH-SY5Y

4.2.4.2.1 Western Blot

Samples were prepared identically to the BE(2)-C cells described in section 4.2.4.1.1 above. The SH-SY5Y cells were thawed in a 37˚C water bath for approximately 2 minutes and transferred to 15mL centrifuge tube. 1mL of warmed maintenance media (table 4.6) is added to the cryovial to collect residue cells and transferred to the cell suspension drop-wise to minimise osmotic shock. The cells are centrifuged for 4 minutes at 300 *xg* and re-suspended in 1mL of media allows for cell count with a haemocytometer (Appendix B). SH-SY5Y cells were maintained for 7 days, before plating on 24-well plates for experiment preparation. Again, at a density of 225,000 cells/well the SH-SY5Y cells detached and the methodology was altered to plate the cells at 10,000

cells/cm², on 25cm² flasks, as previously optimised, and comparable cell numbers to other cell samples were collected (see table 4.6).

4.2.4.2.2 Mass Spectrometry

High density cell samples were collected to analyse the presence of SNAP-25 in the SH-SY5Y cells and optimise the methodology of mass spectrometry. The undifferentiated cells were plated and proliferated to collect a cell sample with approximately 1 x 10_6 cells for analysis. The number of cells available were significantly less than the BE(2)-C cells, but higher than the concentration used for Western Blot. This was intended to be a significant enough increase to be able to detect SNAP-25 in the SH-SY5Y cells using mass spectrometry. The cells were collected in triethylammonium bicarbonate (TEAB), following the methodology in section 4.2.6, and stored at -80˚C before analysis. The method of analysis using mass spectrometry is documented in Chapter 7 describing the approach to trialling selected detection methods.

SH-SY5Y Neuroblastoma Cell Line				
Method	Plating Density	Sample Collection	Maintenance Media 40mL	
Western Blot	10,000 cells/cm² 250,000 cells/ 25cm² flask 10mL media	3 flasks in 60µl of RIPA buffer per sample	EMEM (EBSS) Hams F12 Non-essential amino acids	16.6mL 16.6mL 400µl
Mass Spectrometry	10,000 cells/cm² 250,000 cells/ 25cm² flask 10mL media	4 flasks in 100µl of TEAB buffer	Glutamine Heat inactivated Foetal Bovine Serum (FBS) Penicillin/Streptomycin	400µl 5.6mL 400µl

Table 4.6: SH-SY5Y Neuroblastoma Cell Line Plating Density and Maintenance Media Required for SNAP-25 Expression Analysis Experiments. See Appendices for Suppliers and Product Codes.

4.2.4.3 IMR-32

4.2.4.3.1 Western Blot

IMR-32 samples were prepared by applying the same methodology to those of the BE(2)-C and SH-SY5Y cell lines. Cells were plated at 10,000 cells/cm² on 25cm² flasks and maintained for 7 days before collection. The issues with adherence prevented the trial of a cell density of 225,000 cells per well so the comparable cell number (approximately 775,000 cells/sample) was collected in each IMR-32 sample. The 7-day maintenance period incorporated a media change on day 3 and passaging of cells on day 5. The use of trypsin is not required as the cells detach on movement of the culture-ware and samples were prepared for Western Blot analysis of SNAP-25 expression (see table 4.7).

4.2.4.3.2 Mass Spectrometry

High density cell samples were collected to analyse the presence of SNAP-25 in the IMR-32 cells for mass spectrometry analysis. The undifferentiated cells were plated and proliferated to collect a cell sample with approximately 3 x 10_6 cells for analysis, to increase the likelihood of detection of SNAP-25. The cells were collected in triethylammonium bicarbonate (TEAB), following the methodology in section 4.2.6, and stored at -80˚C before analysis. The methodology of analysis using mass spectrometry is documented in Chapter 7, which describes the approaches to screening detection methods.

IMR-32 Neuroblastoma Cell Line				
Method	Plating Density	Sample Collection	Maintenance Media 40mL	
Western Blot	10,000 cells/cm² 250,000 cells/ 25cm² flask 10mL media	3 flasks in 60µl of RIPA buffer per sample	RPMI 1640 Heat inactivated FBS Non-essential amino acids Penicillin/Streptomycin	31.2mL 8mL 400µl 400µl
Mass Spectrometry	10,000 cells/cm² 250,000 cells/ 25cm² flask 10mL media	12 flasks in 100µl of TEAB buffer		

Table 4.7: IMR-32 Neuroblastoma Cell Line Plating Density and Maintenance Media Required for SNAP-25 Expression Analysis Experiments. See Appendices for Suppliers and Product Codes.

4.2.5 iPSC-derived Cell Line Sample Preparation

4.2.5.1 Human Neural Stem Cells

4.2.5.2 Western Blot

The slow growth of the hNSCs only allowed for limited wells and subsequent reduced sample availability for analysis of SNAP-25 expression, so three separate samples of three wells at 225,000 cells/well were collected (675,000 cells/sample/60µl buffer). Cells were collected as described in section 4.2.6 below.

4.2.5.3 Mass Spectrometry

The limited cell availability prevented the ability to collect samples for mass spectrometry analysis of this cell line. Until results were confirmed for cell line suitability by means of SNAP-25 expression or sensitivity to toxin, further purchase of cell line stock was delayed.

Human Neural Stem Cell Line				
Method	Plating Density	Sample Collection	Maintenance Media 40mL	
Western Blot	112,500 cells/cm² 225,000 cells/well on 24-well plates 500µl media	3 wells in 60µl of RIPA buffer per sample 775,000 cells/sample	NeuroSure Neural Stem Cell Medium #ASM-4011 Applied Stem Cell Penicillin/ Streptomycin	x1 (40mL) 400µl
Mass Spectrometry	Not Applicable			

Table 4.8: Human Neural Stem Cell Line Plating Density and Maintenance Media Required for SNAP-25 Expression Analysis Experiments.

4.2.5.4 ReproCell ACH and DA Cell Lines

4.2.5.4.1 Western Blot

Plates were prepared for the plating of the ACH and DA cell lines, as described in Chapter 3, coating in Poly-L-Orthinine and the supplier provided coating solution before plating. The cells were plated at 225,000 cells/well and cultured for 19 days before sample collection. Media changes were carried out every 2-3 days and samples were collected on day 19 of culture as described in section 4.2.6 below.

4.2.5.4.2 Mass Spectrometry

Only two vials were purchased of the differentiated Reprocell ACH and DA cell lines. SNAP-25 expression is analysed firstly by Western Blot to assess the presence and consistency of expression of SNAP-25 and the results of these experiments will justify further analysis using mass spectrometry. The methodology for mass spectrometry is being optimised at this stage, so to use an expensive, differentiated cell line before the method is working would not be economically beneficial to the project.

ReproCell ACH and DA Cell Lines				
Method	Plating Density	Sample Collection	Maintenance Media 40mL	
Western Blot	112,500 cells/cm² 225,000 cells/well on 24-well plates 500µl media	3 wells in 60µl of RIPA buffer per sample 775,000 cells/sample	Maintenance Media Additive 'A' Penicillin/ Streptomycin	40mL 720µl (ACH) 520µl (DA) 400µl
Mass Spectrometry	Not Applicable			

Table 4.9: ReproCell ACH and DA Cell Line Plating Densities and Maintenance Medias required for SNAP-25 Expression Analysis Experiments.

4.2.5.5 iCell Neurons

4.2.5.5.1 Western Blot

Culture surfaces were prepared by coating 24-well plates in 0.1% Poly-L-Orthinine solution (250µl/well) and following incubation at 37˚C for 1 hour, coated in matrigel coating solution for 30 minutes before use (refer to table 3.8, Chapter 3). Cells were thawed according to the methodology described in section 3.2.7 and plated at a density of 225,000 cells/well (500µl media/well). Half media changes were carried out every 2-3 days, before cell collection. Cells were collected at both 7 and 14-day time points to analyse the changes in expression of SNAP-25 (see section 4.2.6).

4.2.5.5.2 Mass Spectrometry

iCell neurons were plated as described above and grown for 7 days before collection for mass spectrometry analysis. Two samples of 775.000 cells/sample were collected in 60µl of TEAB with protease inhibitors and stored at -80˚C before analysis.

iCell Neurons Cell Line				
Method	Plating Density	Sample Collection	Maintenance Media 100mL	
Western Blot	112,500 cells/cm² 225,000 cells/well on 24-well plates 500µl media	3 wells in 60µl of RIPA buffer per sample	Maintenance Media Supplement Penicillin/ Streptomycin	98mL 2mL 400µl
Mass Spectrometry	112,500 cells/cm² 225,000 cells/well on 24-well plates 500µl media	3 wells in 60µl of TEAB buffer		

Table 4.10: iCell Neurons Cell Line Plating Density and Maintenance Media required for SNAP-25 Expression Analysis Experiments.

4.2.6 Collecting Cells for Western Blot and Mass Spectrometry Analysis

Lysate samples from each of the investigated cell lines were collected following the same procedure. To maximise protein concentration, as SNAP-25 is a low abundance protein which may be challenging to detect, three wells per condition, in which 225,000 cells/well were plated, were collected in 60µl (20µl/well consistently) of RIPA buffer with protease inhibitors (table 4.11). When collecting from flasks, for the neuroblastoma cell lines, the cells were centrifuged to form a pellet which was re-suspended in a comparable volume (225,000 cells/20µl) of RIPA buffer with protease inhibitors.

To detach adherent cells from the plates, the plate, buffer and collection tubes are placed on a tray of ice in order to prevent degradation of the proteins. The media is removed and the wells washed three times with 1x PBS pH7.4 (Life Technologies #10010-23) for 5 minutes. The RIPA buffer with inhibitors is added to the cells in each of the wells, and using a pipette tip, the cells are scraped from the bottom of the well. All cells and buffer, including the bubbles generated by scraping, are collected into a 0.5mL centrifuge tube and stored on ice. The samples are vortexed three times over a period of 15 minutes and then centrifuged at 4˚C at 3000 *xg* for 15 minutes. The supernatant lysate is then transferred to a new tube to discard any pelleted cell debris, and then stored at -80˚C until analysis. This is not necessary for the neuroblastoma cell line sample collection, where the BE(2)-C and SH-SY5Y cells are detached from the flasks using trypsin. The dissociation process is neutralised with the addition of maintenance media containing serum and the cells are centrifuged at 300 *xg* for 4 minutes. To ensure all trypsin is removed from the cells, a repeated wash was carried out; discarding the trypsin supernatant, re-suspending the cells in 1mL of fresh media and repeating the centrifuge process. Subsequently, to remove all media trace from the cells, the cell pellet is re-suspended in PBS, then centrifuged; repeating this process three times. Finally, the cells are suspended in RIPA buffer, or TEAB for mass spectrometry, with protease inhibitors. The IMR-32 cell line does not require the use of trypsin, so the cells are collected following three washes in PBS as previously described.

Solution	Components	Concentration
RIPA Buffer	NaCl	150mM
	NP40	1%
	TRIS pH 7.5	50mM
	EDTA	1.5mM
	Sodium Deoxycholate	1g/100mL
	SDS	0.1g/100mL
TEAB Buffer	Triethylammonium bicarbonate	100mM
	SDS	0.1%

Table 4.11: RIPA and TEAB Cell Collection Buffer Compositions.

4.2.7 Protocol for Western Blot

The protein lysates from each cell line are analysed by Western Blot for the expression of SNAP-25 (criteria 2). This detection method is used as it provides an indication of SNAP-25 presence in a sample using an anti-SNAP25 antibody, detecting whole SNAP-25 protein bands bound to a membrane; allowing quantification of expression. This indicates the level of abundance of SNAP-25 protein in each cell line sample.

Samples were mixed 1:2 in LDS sample buffer (Life Technologies). The loading buffer indicates migration of the proteins through the gel. 25µl of each sample was loaded onto on 12% TGX Protean gels, with 1 well (10µl/well) of BioRad Dual Colour Markers; a molecular marker ladder for identifying protein bands (see Appendix C). The gel was loaded into the BioRad Tetra system, submerged in running buffer (see table 4.13) and run at 200V for 30 minutes; until the LDS sample buffer reached the base of the gel. The proteins are separated in the gel according to molecular weight and then transferred to a nitrocellulose membrane (blot) in transfer buffer (table 4.13) (See Appendix D for detailed protocol). The membrane is then blocked, in 20mL 5% milk powder (see table

4.13) for 30 minutes; this procedure blocks nonspecific binding sites on the membrane, contributing to clearer blots and decreased background signal and more specific binding of the antibody to the protein of interest. To ensure the best possible chance of clearly detecting the low abundant SNAP-25 protein, it was decided that to separate both the actin and SNAP-25 analysis on the membrane would be beneficial to ensure cleaner blots and more specific detection. The membrane was cut in half at the 37kDa marker; clearly separating the SNAP-25 (25kDa) and Actin (42kDa) and each half was incubated in the corresponding antibodies. Following membrane blocking, the blots were incubated overnight in the primary antibodies; anti-SNAP rabbit polyclonal at a concentration of 1:1000 and anti-Beta-Actin rabbit polyclonal at a concentration of 1:20000 (see table 4.12, also refer to Appendix E). The membranes were then washed in 1x TBS three times before addition of the secondary antibody. The blots were incubated in HRP-anti-rabbit, 1:20000 in 5% milk solution for 1 hour. As both primary antibodies are rabbit polyclonal the two blots are incubated in the same secondary antibody. Results were generated, revealing protein bands on film with enhanced chemiluminescence (ECL) (see Appendix G for detailed protocol). Quantification of the intensity of the protein bands was performed using ImageJ software (refer to Appendix H).

Antibody	Provider	Concentration
Rabbit anti-SNAP-25 polyclonal antibody	Synaptic Systems #111 002	1:1000
Mouse anti-SNAP-25 monoclonal antibody	Synaptic Systems #111 111	1:1000
Mouse anti-SNAP-25 monoclonal antibody	Synaptic Systems #111 011	1:1000
Rabbit anti-SNAP-25 polyclonal antibody	Sigma, #S9684	1:1000
Rabbit Beta-Actin (housekeeping protein) antibody	Synaptic Systems #251 003	1:10000
AffiniPure Donkey Anti-Rabbit IgG (H+L)	Jackson ImmunoResearch #715005152	1:20000
AffiniPure Donkey Ant-Mouse IgG (H+L)	Jackson ImmunoResearch #715005150	1:20000

Table 4.12: Table of Antibodies used throughout the Project for the Detection of SNAP-25 and Beta-Actin.

Solution	Components	Concentrations	Volumes/ Weights (1 litre)
Running Buffer	BioRad SDS 10x buffer Distilled water	10% 90%	100mL 900mL
Transfer Buffer	TRIS Glycine Methanol Distilled Water		3.03g 14.4g 200mL 800mL
TBS Buffer 10x	TRIS NaCl Distilled water	Adjust pH to 7.6 adding hydrochloric acid	61g 90g 1 litre
Blocking Buffer	Dried milk 1 x TBS		1g 20mL
Antibody solution	Dried milk 1x TBS		2.5g 50mL

Table 4.13: Western Blot Reagents

4.2.8 Protocol for Mass Spectrometry

Several approaches to sample preparation were trialled in the optimisation of the mass spectrometry methodology, discussed in Chapter 7. The iCell neurons were the first cell line analysed, alongside a reference peptide for the C-terminus of SNAP-25, with varying results which lead to the requirement for method adaptation. The high cell density analysis of the neuroblastoma cell lines followed a further adapted protocol in the attempt to detect the presence of SNAP-25. An overview of the methodologies are described below, and in complete detail in Chapter 7.

4.2.8.1 iPSC-derived Cell Line Analysis with Mass Spectrometry

4.2.8.1.1 Initial methodology

Collected cell samples in TEAB and SDS were sonicated before being washed through 30kDa spin-filters to remove the larger proteins from the analysed sample. The sample was lyophilised overnight then washed following the C18 protocol which allows for reversed solid phase extraction of peptides and concentrates and de-salts samples. The cell sample was digested with trypsin before analysis, to breakdown the proteins into detectable peptides and analysed with the mass spectrometer. (See Chapter 7)

4.2.8.1.2 Adapted methodology

The size of the filters was changed from 30kDa to 10kDa, to specifically target the capture of the peptides of lower sized proteins. A different reagent was used for the cleavage of peptides for detection, altering trypsin to Lys-C, which cleaves peptides at the C-terminus of lysine. This was altered to assess any difference in detection if the proteins are broken down into different sized peptides. The C18 protocol was completed as used previously. (See Chapter 7).

4.2.8.2 Neuroblastoma Cell Line Analysis with Mass Spectrometry

With abundant protein in the high cell density samples, the mass spectrometry approach was altered further, introducing the use of OFFGEL fractionation and a FASP preparation protocol. The FASP approach allows for the solubilisation of samples in SDS and digestion in trypsin, and the C18 clean-up was carried out after this procedure. OFFGEL Fractionation allows for the separation of peptides, into fractions, for a more specific detection of peptides of interest.

4.3 Results – Analysis of SNAP-25 Expression

4.3.1 Differentiation of Neuroblastoma Cell Lines

The intention of experimenting with differentiation protocols was to investigate potential improvement of the expression of SNAP-25 following a neuronal differentiation protocol. The cells were immunoassayed for the presence of neuronal cell markers to indicate potential neuronal lineage as a result of differentiation and also analysed with Western Blot for SNAP-25 expression.

4.3.1.1 Differentiation with Vasoactive Intestinal Peptide

4.3.1.1.1 BE(2)-C Cell Line

BE(2)-C cells were successfully incubated with VIP for 9 days and remained adherent and visually healthy in both the VIP and the control buffer medias. The BE(2)-C cells were then fixed for immunocytochemistry analysis.

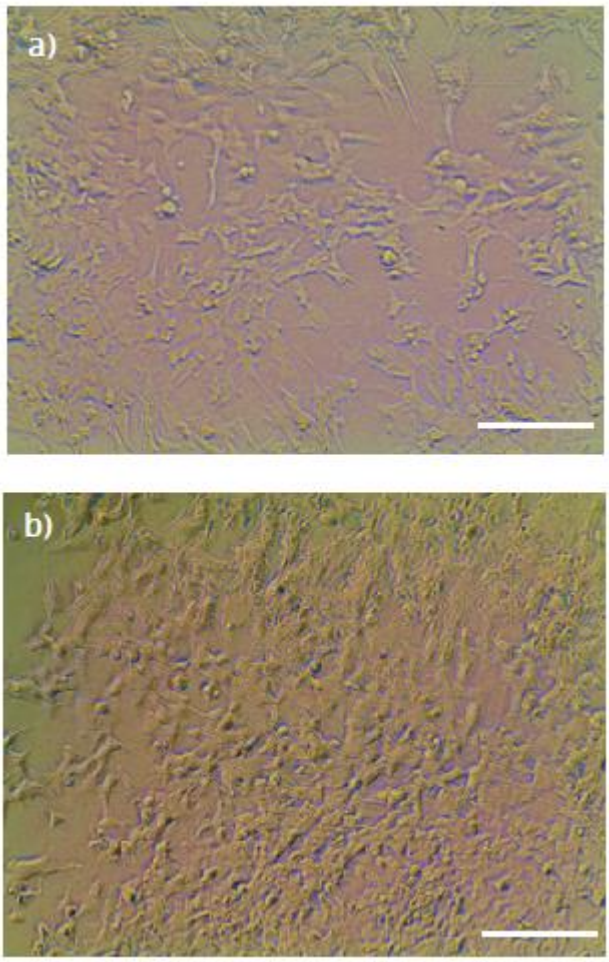


Figure 4.2: BE(2)-C cells in Vasoactive Intestinal Peptide*. Phase contrast images captured on day 7 of differentiation* ***(a)*** *BE(2)-C in VIP* ***(b)*** *BE(2)-C water control. x20 field of view, scale bar 100µm.*

Proliferation of the BE(2)-C cells ceased as a result of addition of VIP to the maintenance media, and the cells began to develop neurite extension, as shown in figure 4.2a. Day 7 is shown in this image, and the morphology of the cells shown in figure 4.2a remained the same at day 9, when the cells were fixed for immunostaining. The observations of the cells indicate that VIP treatment induces morphological changes in the BE(2)-C cell line, possibly indicative of neuronal differentiation.

Criteria	BE(2)-C
Extension of neurite processes	✓
Increase in size of cells	x
Inhibition of cell proliferation/division	✓

Table 4.14: Criteria in the assessment of differentiation with VIP for the BE(2)-C cell line.

The defined criteria of an increase in size of cells; indicative of neuronal differentiation (Wallace and Das, 1983) cannot be applied to the observations of the BE(2)-C cells in culture. Further analysis with immunostaining for beta-tubulin, ChAT and MNR2 was carried out to indicate whether VIP has induced differentiation of the BE(2)-C cells into neuronal cell types; cholinergic (ChAT) and motor neurons (Beta-tubulin and MNR2).

4.3.1.1.1.1 Beta-Tubulin Immunostaining of VIP Differentiated BE(2)-C Cells

The cells shown in the images in figure 4.3 were stained with Beta-III-tubulin markers and images were captured using a green filter; highlighting the presence of Beta-III-tubulin. Beta-III-Tubulin is abundant in the BE(2)-C cells differentiated in VIP, and as an early neuronal marker, detection indicates that the cells are demonstrating characteristics of the neuronal phenotype. It is a cytoplasmic neuronal marker, as shown in figure 4.3a.

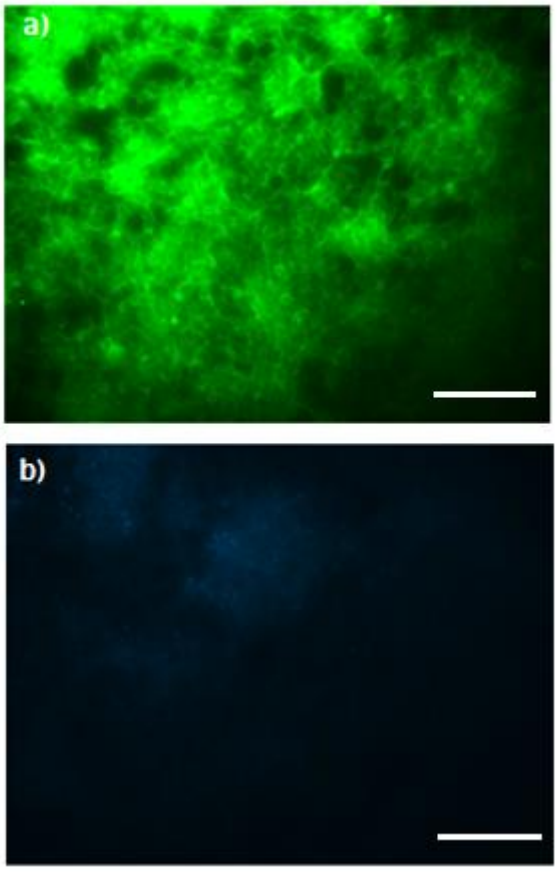


Figure 4.3: Expression of Beta-III-Tubulin in BE(2)-C cells following VIP Incubation. *Immunofluorescent images captured of cells fixed at day 9 of differentiation* ***(a)*** *Beta-tubulin BE(2)-C VIP differentiated cells* ***(b)*** *DAPI blue control BE(2)-C VIP differentiated cells. Images captured at x20 field of view, scale bar 100µm.*

4.3.1.1.1.2 ChAT Immunostaining of VIP Differentiated BE(2)-C Cells

The cells shown in the images in figure 4.4 were stained with an anti-ChAT antibody at a concentration of 1:50 and the images were captured using a red filter, which highlights the presence of ChAT in the BE(2)-C cells. The three images are layered, with both the ChAT captured image and the DAPI staining for the same cells/well. The minimal red staining suggests that there are not any cholinergic neurons in the BE(2)-C VIP differentiated cell population, and any detected red areas are related to unspecific staining. ChAT is present in the cytoplasm of the cells, so if present, the images captured would be expected to be comparable to those shown in figure 4.3 for the analysis of Beta-tubulin. ChAT, at this stage of differentiation is not abundant; or cholinergic neurons are not a resulting cell type following differentiation with VIP.

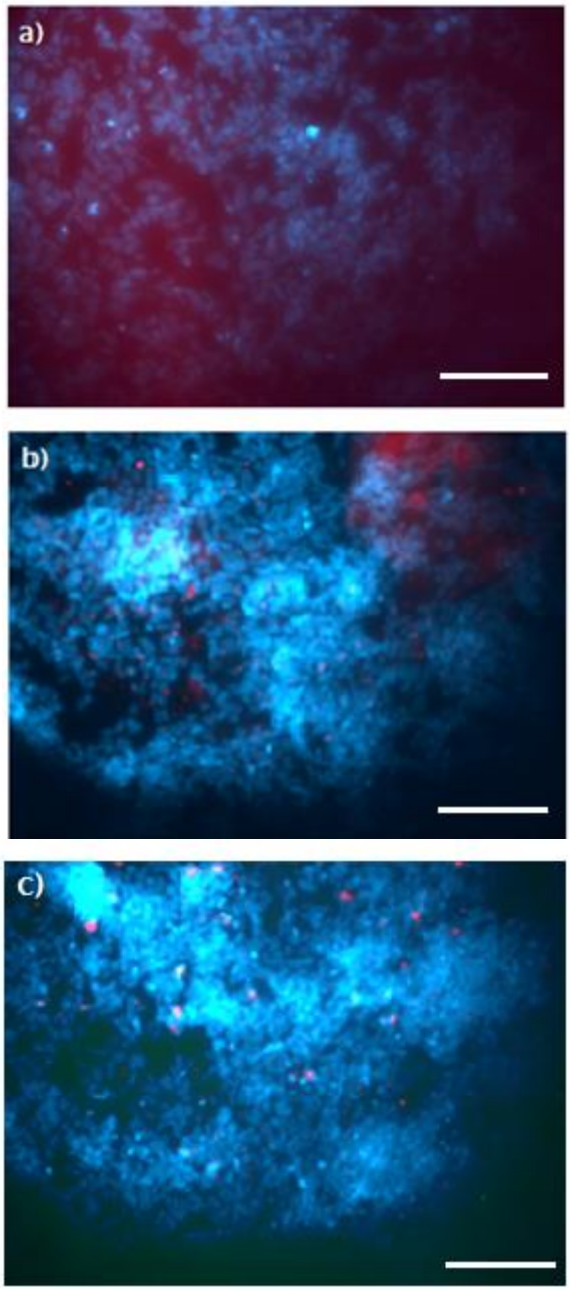


Figure 4.4: Expression of ChAT in BE(2)-C cells following VIP Incubation. *Immunofluorescent images captured of cells fixed at day 9 of differentiation* ***(a)*** *and* ***(b)*** *ChAT BE(2)-C VIP differentiated cells layered image;* ***ChAT*** *red filter image* ***DAPI*** *blue filter image. Two replicate wells shown.* ***(c)*** *Negative control well showing unspecific staining. Images captured at x20 field of view, scale bar 100µm.*

4.3.1.1.1.3 MNR2 Immunostaining of VIP Differentiated BE(2)-C cells

The cells shown in the images in figure 4.5c were stained with undiluted MNR2 antibody. Not diluting the antibody yielded the clearest results following methodology optimisation. Images were captured with a green filter, (DAPI shown in blue), which highlights the presence of MNR2 in the BE(2)-C cells. MNR2 is expressed in the cell nucleus, and expression of MNR2 in neural cells initiates somatic motor neuron differentiation (Tanabe, William et al. 1998). The layered images in figure 4.5b show that with 1:5 dilution of antibody, the staining remains unspecific and does not show a clear indication of the presence of MNR2 in the BE(2)-C cells.

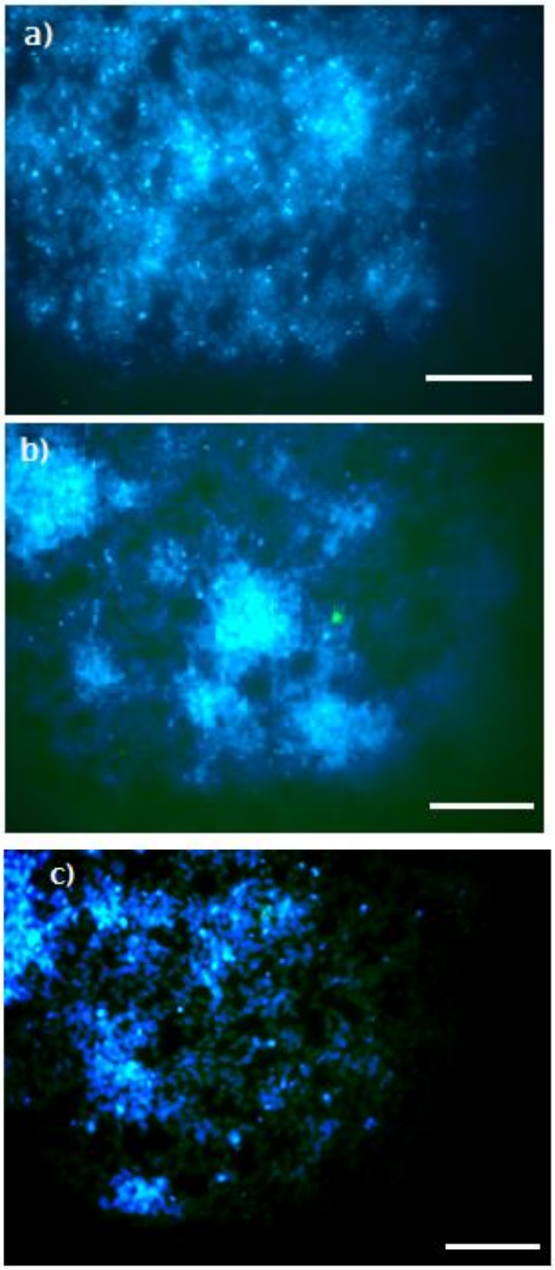


Figure 4.5: Expression of MNR2 in BE(2)-C cells following VIP Incubation. *Immunofluorescent images captured of cells fixed at day 9 of differentiation* ***(a)*** *negative control well* ***(b)*** *MNR2 BE(2)-C VIP differentiated cells layered image;* ***MNR2*** *green filter image* ***DAPI*** *blue filter image, 1:5 antibody* ***(c)*** *MNR2 BE(2)-C VIP differentiated cells layered image;* ***MNR2*** *green filter image* ***DAPI*** *blue filter image, undiluted antibody. Two wells shown. Images captured at x20 field of view, scale bar 100µm.*

4.3.1.1.2 SH-SY5Y Cell Line

The SH-SY5Y neuroblastoma cells also responded well to the VIP, when observing the cells in culture. The cells incubated for 9 days without issue and were stained for fluorescent analysis of neuronal differentiation with VIP. .

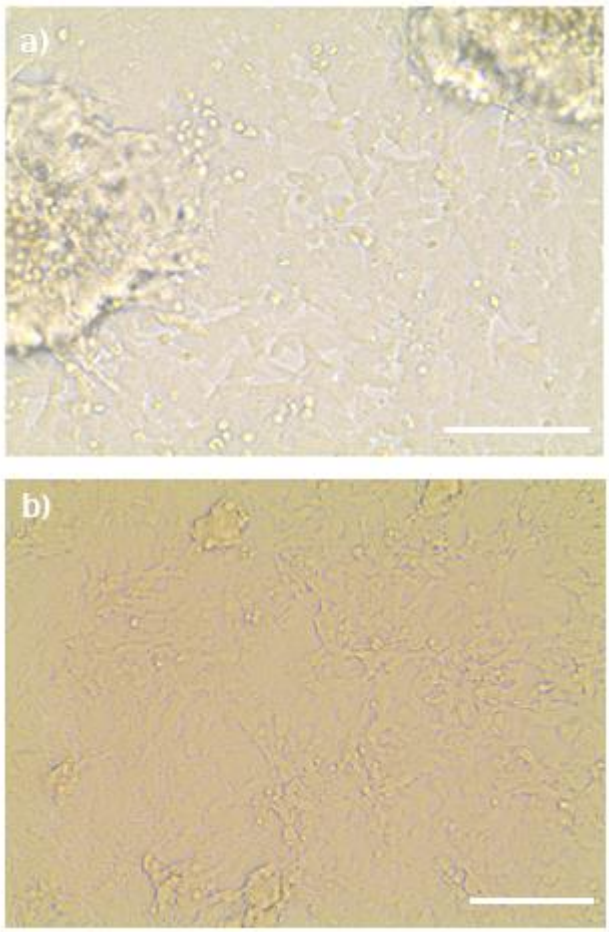


Figure 4.6: SH-SY5Y cells in Vasoactive Intestinal Peptide. *Phase contrast images captured on day 7 of differentiation* ***(a)*** *SH-SY5Y in VIP x20 field of view* ***(b)*** *SH-SY5Y DMSO control x 20 field of view, scale bar 100µm.*

Addition of VIP to the maintenance media for the SH-SY5Y cells ceased cell proliferation and the morphology of the cells was visibly altered. When comparing the DMSO treated cells and the VIP treated cells in figure 4.6a, the VIP incubated cells have adhered with extended neurite growth, but have also taken a fibroblast-like formation, with a high density of 'clumped' cells. The images were captured on day 5 of VIP differentiation, and the morphology of the cells remained consistent until day 9, when fixed for immunostaining.

Criteria	SH-SY5Y
Extension of neurite processes	✓
Increase in size of cells	x
Inhibition of cell proliferation/division	✓

Table 4.15: Criteria in the assessment of differentiation with VIP for the SH-SY5Y cell line.

Much like the BE(2)-C cells, the SH-SY5Y cells did not display an increase in cell size, expected of neuronal differentiation, but the two criteria of extension of neurites and inhibition of cell division were met. Analysis of differentiation with immunocytochemistry is for the SH-SY5Y cell line is outlined below, using the same antibodies as the BE(2)-C cells; Beta-tubulin, CHAT and MNR2.

4.3.1.1.2.1 Beta-tubulin Immunostaining of VIP Differentiated SH-SY5Y Cells

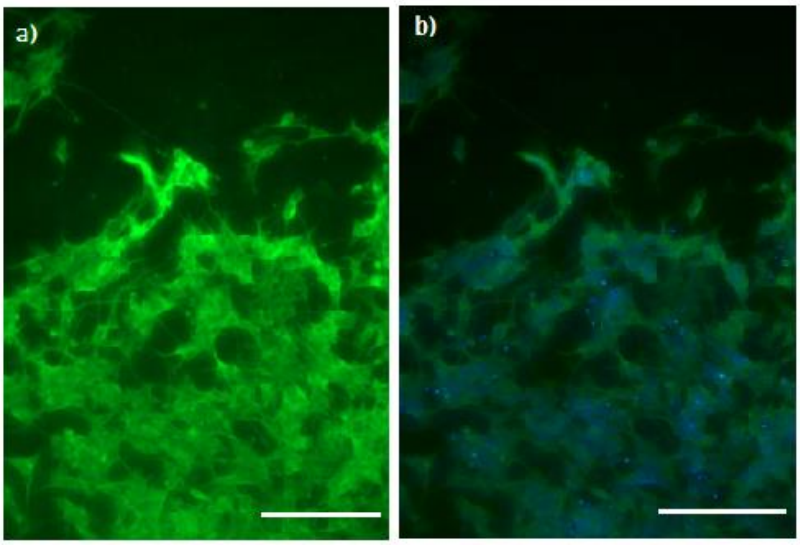


Figure 4.7: Expression of Beta-III-Tubulin in SH-SY5Y cells following VIP Incubation. *Immunofluorescent images captured of cells fixed at day 9 of differentiation* ***(a)*** *Beta-tubulin (green) SH-SY5Y VIP differentiated cells* ***(b)*** *Beta-tubulin (green) SH-SY5Y VIP differentiated merged image; Beta-Tubulin green filter image* ***DAPI*** *blue filter image . Images captured at x40 field of view, scale bar 100µm.*

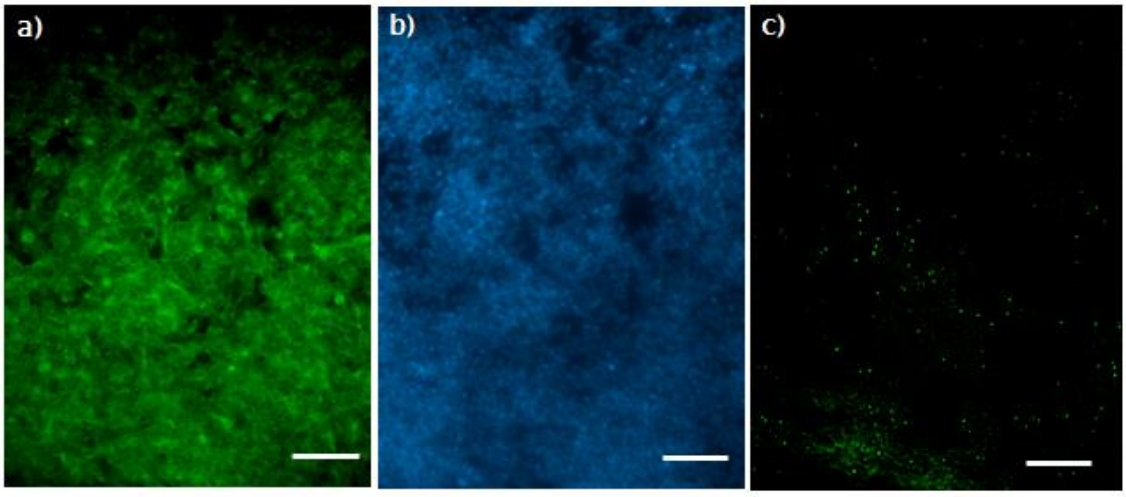


Figure 4.8: Expression of Beta-III-Tubulin in SH-SY5Y cells following VIP Incubation*.* ***(a)*** *Beta-tubulin (green) SH-SY5Y VIP differentiated cells* ***(b)*** *DAPI (blue) SH-SY5Y VIP differentiated cells; image captured in the same position in the well as image a.* ***(c)*** *negative control well. Images captured using a fluorescent microscope, at x20 field of view, scale bar 100µm.*

Figures 4.7 and 4.8 above show images of Beta-tubulin staining of the SH-SY5Y cells. Beta-III-tubulin is abundant in cytoplasm of the SH-SY5Y cells and detection indicates neuronal characteristics. This marker can clearly be seen in the SH-SY5Y cells, with clear green highlight of the presence of Beta-III-tubulin. This suggests that the cells, at this point of the differentiation process, are of neuronal lineage.

4.3.1.1.2.2 ChAT Immunostaining of VIP Differentiated SH-SY5Y Cells

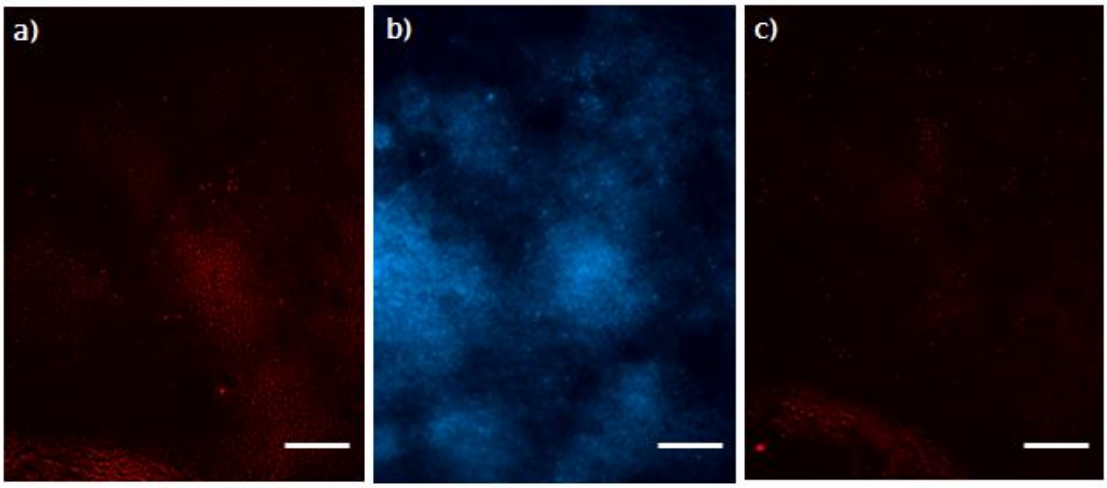


Figure 4.9: Expression of ChAT in SH-SY5Y cells following VIP Incubation. (a) *ChAT (red) SH-SY5Y VIP differentiated cells* ***(b)*** *DAPI (blue) SH-SY5Y VIP differentiated cells; image captured in the same position in the well as image a.* ***(c)*** *negative control well. Images captured using a fluorescent microscope, at x20 field of view, scale bar 100µm.*

SH-SY5Y cells were fixed in PFA, as described in the methodology, at day 9 of differentiation in VIP. Presence of ChAT is shown in red in the image in figure 4.9a above, with the DAPI staining for the same well in figure 4.9b. The negative control (figure 4.9c) indicates that the red staining shown in image A is likely to be unspecific staining opposed to detection of ChAT, determining the unlikely presence of cholinergic neurons in the differentiated SH-SY5Y cells.

4.3.1.1.2.3 MNR2 Immunostaining of VIP Differentiated SH-SY5Y Cells

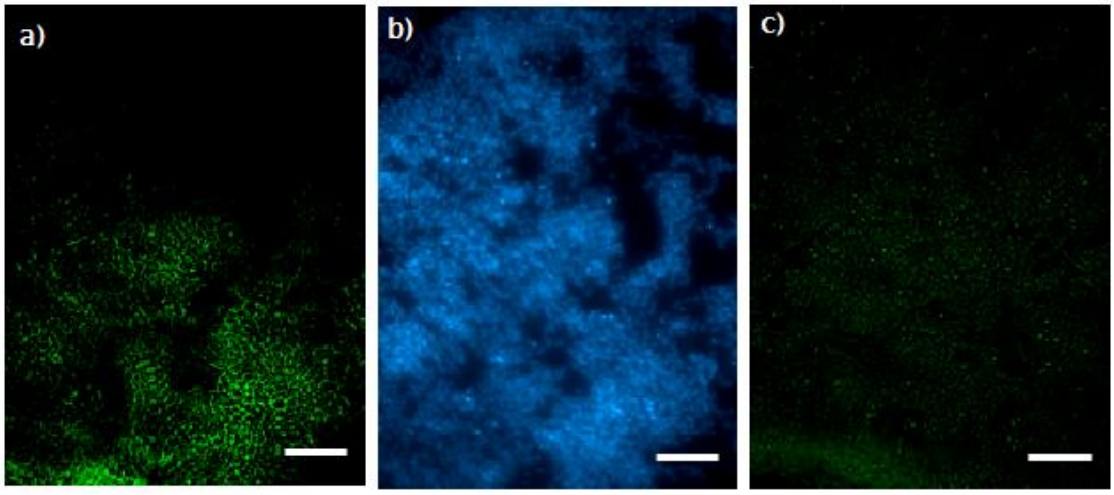


Figure 4.10: Expression of MNR2 in SH-SY5Y cells following VIP Incubation. *(a) MNR2 (green) SH-SY5Y VIP differentiated cells (b) DAPI (blue) SH-SY5Y VIP differentiated cells; image captured in the same position in the well as image a. (c) negative control well. Images captured using a fluorescent microscope, at x20 field of view, scale bar 100µm.*

The immunostaining for the expression of MNR2 was more successful when analysing the SH-SY5Y cell line than the results for the BE(2)-C. Figure 4.10a shows nuclear staining, in green, as a result of incubation in undiluted MNR2 antibody. The control well shown in figure 4.10c shows the unspecific staining, but the detection of MNR2 appears to be more prominent in figure 4.10a suggesting expression of MNR2 in this cell line. This is only a suggestion however, as the comparison to undifferentiated cells is not shown and this level of expression could be consistent without the incubation with VIP. These results suggest that a motor neuron marker is expressed.

4.3.1.1.3 IMR-32 Cell Line

The IMR-32 cells did not respond well to incubation with VIP, with all the cells detaching from the plate almost immediately after addition. Analysis of cell suspension with trypan blue at day 3 of incubation indicated high cell death. The

concentration of VIP used was comparable to the BE(2)-C and SH-SY5Y and was trialled various times, each with the cells detaching before day 9 of the experiment (see figure 4.11 below).

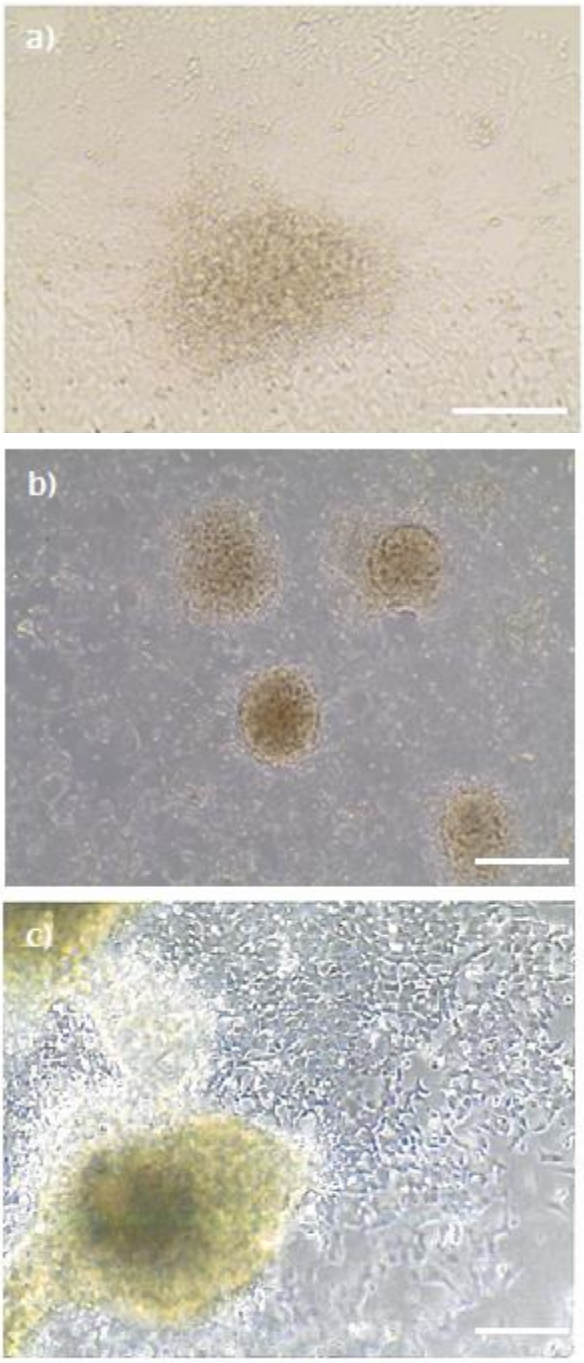


Figure 4.11: IMR-32 Cells in Vasoactive Intestinal Peptide. *Phase contrast images captured after addition of VIP* ***(a)*** *IMR-32 water control* ***(b)*** *IMR-32 2-hours in VIP* ***(c)*** *IMR-32 24-hours in VIP water control. Images captured at x20 field of view, scale bar 100µm.*

Images for the BE(2)-C and SH-SY5Y cell lines were captured at day 7 of VIP differentiation, but the IMR-32 cells consistently detached and could not be continued in culture for the same period of time. This proved challenging in the approach to immunostaining for marker expression, and when plated for experiments, the IMR-32 cells would detach from the plate and would die between days 3-4. This prevented the collection of comparable data to that observed for the other neuroblastoma cell lines. It has proven to be problematic with regard to maintaining the IMR-32 cell line and the adherence to experimental design is difficult to manage. This was considered when assessing the continuation of analysis of the IMR-32 cell line.

Criteria	IMR-32
Extension of neurite processes	n/a
Increase in size of cells	n/a
Inhibition of cell proliferation/division	n/a

Table 4.16: Criteria in the assessment of differentiation with VIP for the IMR-32 line. *Due to the detachment of the cells the criteria could not be applied or assessed for the IMR-32 cell line, so is observed as n/a.*

With reference to table 4.16 above, the IMR-32 did not adhere to any of the defined criteria that indicate neuronal differentiation. The cells were not adherent; in several attempts to culture the cells with the addition of VIP, preventing the observation of extended neurite processes, increase in the size of the cells or obvious inhibition of cell proliferation. The concluding results from the analysis of the IMR-32 cell line and its response to differentiation with VIP suggest that the cell line is not compatible with this protocol. The results for incubation with RA are discussed in the following section 4.3.1.2.3.

4.3.1.2 Retinoic Acid Differentiation of Neuroblastoma Cell Lines

Analysis of changes in morphology, and possible indication of differentiation through the detection of neuronal markers was carried out to assess the efficiency of retinoic acid incubation as a differentiation method.

Several attempts at retinoic acid differentiation were performed for the BE(2)-C, SH-SY5Y and IMR-32 cell lines, two replicates of the experiments on these cell lines induced cell death within 48 hours. On the third attempt, the cells were healthy until day 6, but then detached and died after a media change. RA is extensively used in neuronal differentiation protocols (Kim, Habiba et al. 2009) (Okada, Shimazaki et al. 2004) so it was assumed to be an issue with the methodology or stock of RA. A new stock of RA was used and successful incubation for the 9 days duration was finally completed, suggesting an issue with the original stock.

4.3.1.2.1 BE(2)-C Cell Line

Addition of RA to the maintenance media for the BE(2)-C cells initiated a cease in cell proliferation and the morphology of the cells was visibly altered. When comparing the DMSO control well and the RA cells in figure 4.12a below, the RA differentiated cells have adhered but extended neurite growth was not as advanced at day 5, in comparison to the VIP differentiated BE(2)-C cells (see figure 4.2a shown previously). The morphology of the cells shown in figure 4.12, captured at day 5 of RA differentiation indicates the same morphology shown on day 9, when fixed for immunostaining.

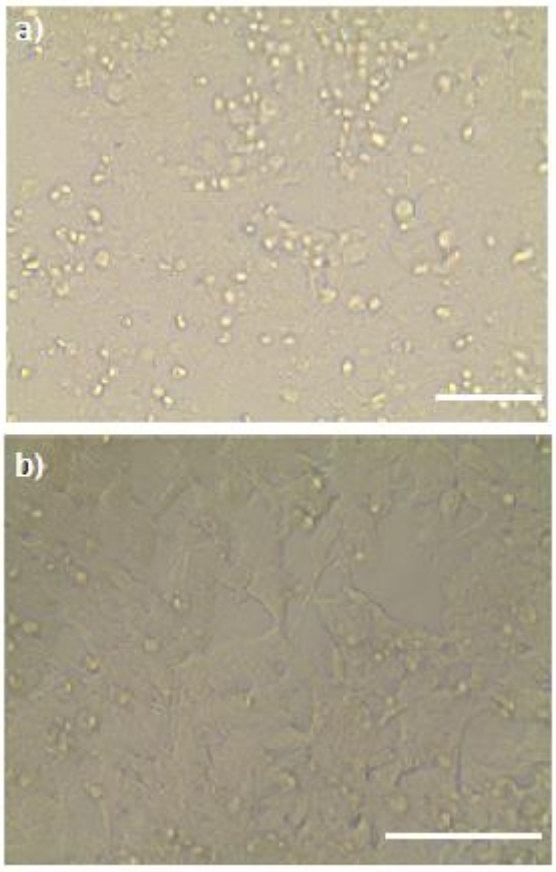


Figure 4.12: BE(2)-C Cells in Retinoic Acid. *Phase contrast images captured on day 5 of differentiation* ***(a)*** *BE(2)-C in RA* ***(b)*** *BE(2)-C DMSO control. Images were captured at x40 field of view, scale bar 100µm.*

Table 4.17 below applies the set criteria for visual analysis of potential differentiation for the BE(2)-C neuroblastoma cell line.

Criteria	BE(2)-C
Extension of neurite processes	x
Increase in size of cells	✓
Inhibition of cell proliferation/division	✓

Table 4.17: Criteria in the assessment of differentiation with RA for the BE(2)-C cell line.

An increase in the size of the cells is clearly observed in the image in figure 4.12a, compared to 4.12b. Repeated experiments demonstrated this increase in size consistently, but visible extension of neurite processes was not apparent in the BE(2)-C cells.

4.3.1.2.1.1 Expression of SNAP-25 by Western Blot Analysis

Following differentiation, samples of the BE(2)-C cells were collected for analysis of SNAP-25 via Western Blot. Figure 4.13 below shows a SNAP-25 blot for BE(2)-C and SH-SY5Y; both undifferentiated and RA differentiated samples. As shown, the expression of SNAP-25 was not detected in the BE(2)-C cells, in both undifferentiated and RA differentiated samples, with the protein concentration available for Western Blot analysis.

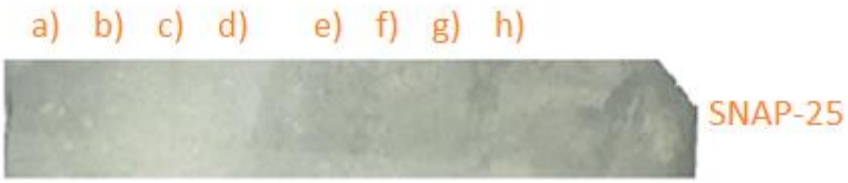


Figure 4.13: SNAP-25 blot analysis of BE(2)-C and SH-SY5Y cells (a) *BE(2)-C undifferentiated cells* ***(b)*** *BE(2)-C RA differentiated cells* ***(c)*** *BE(2)-C DMSO control* ***(d)*** *BE(2)-C water control* ***(e)*** *SH-SY5Y undifferentiated cells* ***(f)*** *SH-SY5Y RA differentiated cells* ***(g)*** *SH-SY5Y DMSO control* ***(h)*** *SH-SY5Y water control.*

4.3.1.2.2 SH-SY5Y Cell Line

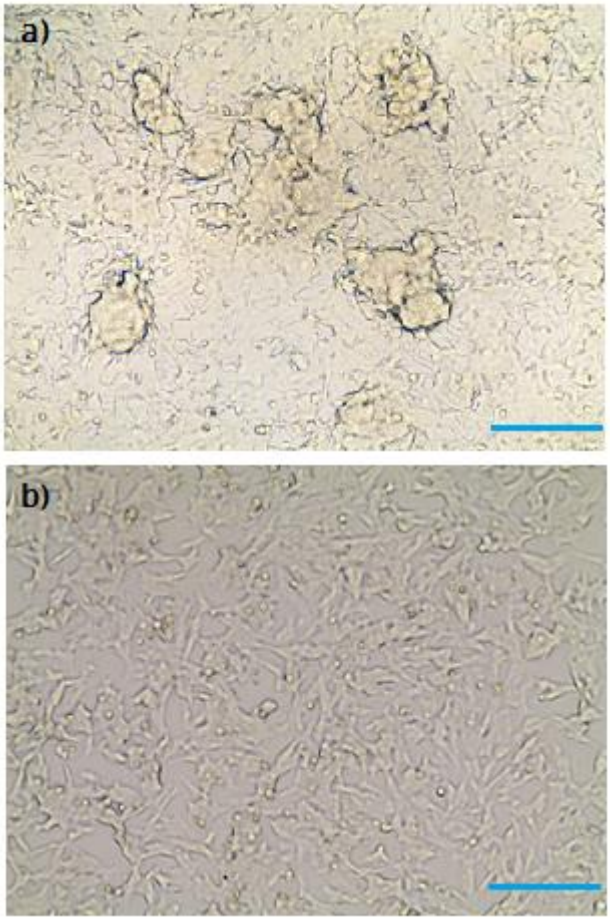


Figure 4.14: SH-SY5Y cells in Retinoic Acid. *Phase contrast images captured on day 5 of differentiation* ***(a)*** *SH-SY5Y in RA* ***(b)*** *SH-SY5Y DMSO control. Images were captured at x40 field of view, scale bar 100µm.*

Addition of RA to the maintenance media for the SH-SY5Y dramatically altered the morphology of the cells, showing aggregated 'clumps' of cells; a morphology which was onset from day 3- 9 of the RA differentiation. RA also initiated a cease in cell proliferation. When comparing the DMSO treated cells (figure 4.14b) and the RA incubated cells in figure 4.14a, the RA treated cells have adhered but extended neurite growth was not as advanced at day 5, in comparison to the VIP incubated SH-SY5Y cells (see figure 4.6a). This is comparable to the observations of RA differentiated BE(2)-C cells. The morphology of the cells shown in figure 4.14, captured at day 5 of RA differentiation indicates the same morphology shown on day 9, when fixed for immunostaining. There was no noticeable

increase in the size of the cells, leaving inhibition of cell proliferation as the only criteria met by the SH-SY5Y cells.

Criteria	SH-SY5Y
Extension of neurite processes	x
Increase in size of cells	x
Inhibition of cell proliferation/division	✓

Table 4.18: Criteria in the assessment of differentiation with RA for the SH-SY5Y cell line.

4.3.1.2.2.1 Expression of SNAP-25 by Western Blot Analysis

The SH-SY5Y cells were collected for analysis of expression of SNAP-25 following differentiation. Undifferentiated cells and cells incubated in retinoic acid for 9 days were collected in RIPA buffer, and analysed via Western Blot for the expression of SNAP-25. Figure 4.15 shows a Western blot analysis for BE(2)-C and SH-SY5Y; both undifferentiated and RA differentiated.

The blot indicates that the BE(2)-C and SH-SY5Y cell lines do not express SNAP-25 at the cell density and concentration provided in the collected samples. The analysis was run with a SNAP-25 control to indicate any issues with Western Blot analysis confirming the observation that SNAP-25 is not abundant or detectable in both the SH-SY5Y and BE(2)-C cell lines, in either untreated or RA treated samples.

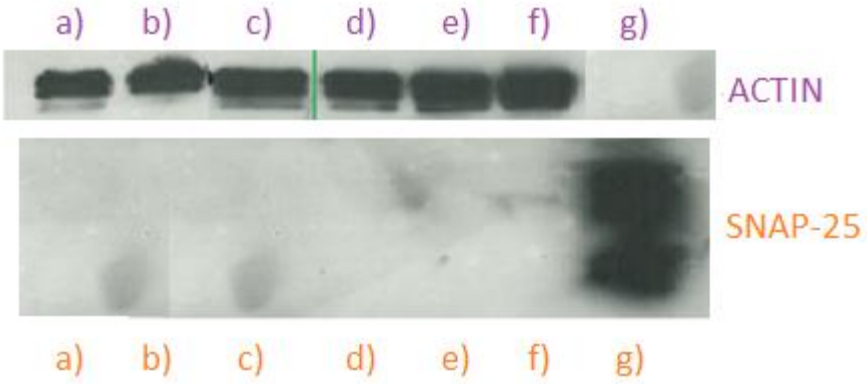


Figure 4.15: Western Blot Analysis of Differentiated Neuroblastoma Cell Lines *(a) SNAP-25 BE(2)-C undifferentiated (a) Actin (b) SNAP-25 BE(2)-C RA differentiated (b) Actin (c) SNAP-25 BE(2)-C DMSO control (c) Actin (d) SNAP-25 SH-SY5Y undifferentiated (d) Actin (e) SNAP-25 SH-SY5Y RA differentiated (e) Actin (f) SNAP-25 SH-SY5Y DMSO control (f) Actin (g) SNAP-25 standard (g) no corresponding Actin as expected. SNAP-25 detected at 25kDa and Beta-Actin at 42kDa.*

Although not well defined, the standard has produced a detectable band for SNAP-25, and indicates that there are no issues with the methodology and that SNAP-25 is not expressed, at a detectable level for Western Blot analysis in either undifferentiated or RA differentiated BE(2)-C or SH-SY5Y cells. The SNAP-25 standards are assessed for specificity and this is discussed in Chapter 7; trialling detection methods.

4.3.1.2.3 IMR-32 Cell Line

Similar results with RA treatment were observed on the addition of RA to the VIP differentiation experiments for the IMR-32 cell line. The cells did not respond well; addition of RA induced the same issues as preciously described, with the cells detaching within 72 hours of differentiation; an extended period in comparison to the VIP incubated IMR-32 cells. Cells were collected, after 72-hours, and added to trypan blue, which indicates the number of viable cells and approximately 95% of the cells were stained blue, indicating cell death. Four squares on the haemocytometer grid were counted, totalling 3575 cells/µl, of

which 185 were viable cells. This is a high cell death on addition of RA, and this was also indicated in the DMSO treated well; 3129 cells/µl, 1792 viable cells.

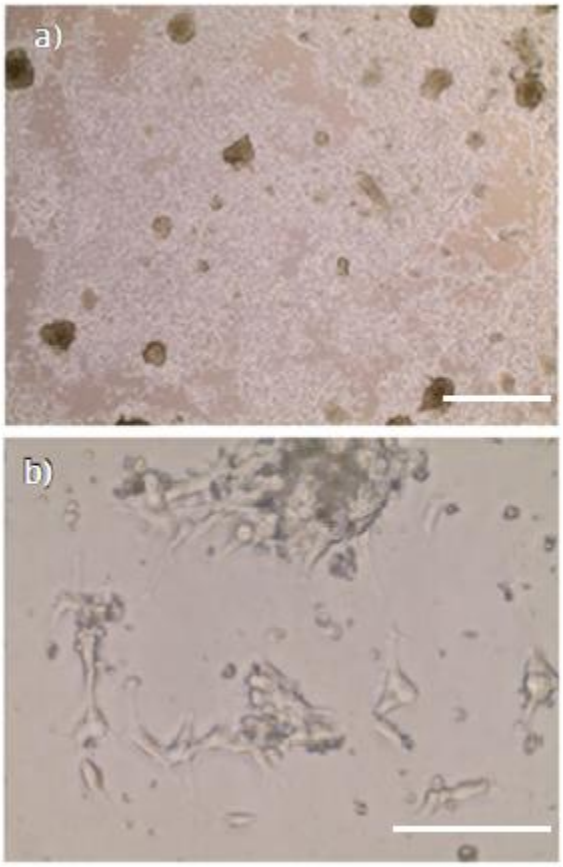


Figure 4.16: IMR-32 cells in Retinoic Acid. *Phase contrast images captured on day 3 of differentiation* ***(a)*** *IMR-32 in RA x20 field of view* ***(b)*** *IMR-32 DMSO control x40 field of view, scale bar 100µm.*

The adherence of the cells was slightly improved in the RA cells compared to those incubated in VIP, but the expected morphology was not shown. The cells were not adherent for long enough to observe extension of neurite processes, or a change in the size of the cells, expected as a result of neuronal differentiation.

Criteria	IMR-32
Extension of neurite processes	n/a
Increase in size of cells	n/a
Inhibition of cell proliferation/division	n/a

Table 4.19: Criteria in the assessment of differentiation with VIP for the IMR-32 line. *Due to the detachment of the cells the criteria could not be applied or assessed for the IMR-32 cell line, so is observed as n/a.*

With reference to table 4.19 above, the IMR-32 did not adhere to any of the defined criteria that indicate neuronal differentiation. The cells were not adherent; in several attempts to culture the cells with the addition of RA, preventing the observation of extended neurite processes, increase in the size of the cells or obvious inhibition of cell proliferation. The concluding results from the analysis of the IMR-32 cell line and its response to differentiation with RA suggests that the cell line is not compatible with this protocol.

4.3.1.2.3.1 Expression of SNAP-25 by Western Blot Analysis

The issues surrounding the survival of the IMR-32 cells prevented the collection of samples for the analysis of expression of SNAP-25 succeeding differentiation. The cell staining with trypan blue indicated that the cells were not viable after 72-hours of RA exposure, rendering it impossible to collect samples at day-9 to compare to the BE(2)-C and SG-SY5Y neuroblastoma cell lines.

4.3.2 Undifferentiated Neuroblastoma SNAP-25 Expression Analysis

This results section is composed of both Western Blot and Mass Spectrometry analysis of undifferentiated cell samples for each of the three neuroblastoma cell lines. Undifferentiated sample analysis was shown previously where figures 4.13 and 4.15 demonstrate Western Blot analysis that includes undifferentiated BE(2)-C and SH-SY5Y cells as a comparable sample to differentiated samples. The analysis of undifferentiated BE(2)-C, SH-SY5Y and IMR-32 neuroblastoma cell lines is discussed in further detail in the following sections.

4.3.2.1 BE(2)-C Cell Line

4.3.2.1.1 Expression of SNAP-25 by Western Blot Analysis

BE(2)-C samples were analysed, alongside the SH-SY5Y and IMR-32 undifferentiated cell lines, for expression of SNAP-25. The cell densities used in each sample were comparable to the iPSC-derived cell lines (775,000 cells/sample). The analysis was carried out three times, on three different collected samples; each with the same result as shown in the blot in figure 4.18 below. Repeated analysis indicates that the BE(2)-C do not express levels of SNAP-25 that are detectable at this cell density in Western Blot.

4.3.2.1.2 Expression of SNAP-25 by Mass Spectrometry Analysis

The protein concentration of the sample of BE(2)-C cells collected for mass spectrometry was analysed using a Direct Detect Concentrator (Merck Millipore). The highest concentration BE(2)-C sample was analysed, shown in figure 4.17 (and referenced in Chapter 7). The protein concentration for the presented and analysed sample was 8.804µg/µl.

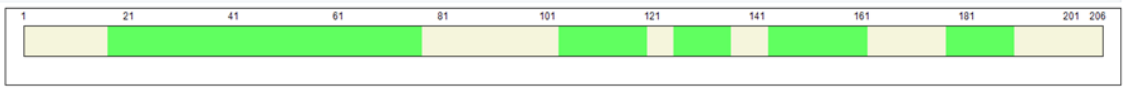


Figure 4.17: Mass Spectrometry SNAP-25 peptides detected in BE(2)-C high cell density sample. *The peptides detected in the 206-amino acid protein are shown in green. 54% peptide coverage was detected.*

The mass spectrometry analysis showed that among the different peptides in the cell sample, peptides corresponding to 54% of the whole SNAP-25 protein were detected. The results of the mass spectrometry analysis indicate different results to those demonstrated in the Western Blot analysis, and show that the BE(2)-C cells do express SNAP-25, with analysis with mass spectrometry capable of demonstrating 54% peptide detection coverage. These results are not comparable in terms of protein concentrations, suggesting that perhaps an increase in cell density and subsequent SNAP-25 protein abundance could improve detection using Western Blot.

4.3.2.2 SH-SY5Y Cell Line

4.3.2.2.1 Expression of SNAP-25 by Western Blot Analysis

Figure 4.18 below shows an example of Western Blot analysis of the neuroblastoma cell lines BE(2)-C, SH-SY5Y and IMR-32. Two replicates for each cell line are shown on the same blot indicating that SNAP-25 cannot be detected in any of the undifferentiated cell lines. Figure 4.15 shown previously shows the undifferentiated samples for the SH-SY5Y cells alongside a SNAP-25 control; confirming the Western Blot methodology capabilities which leads to the suggestion that the SH-SY5Y cell line does not express SNAP-25 at a level detectable by Western Blot at this cell density.

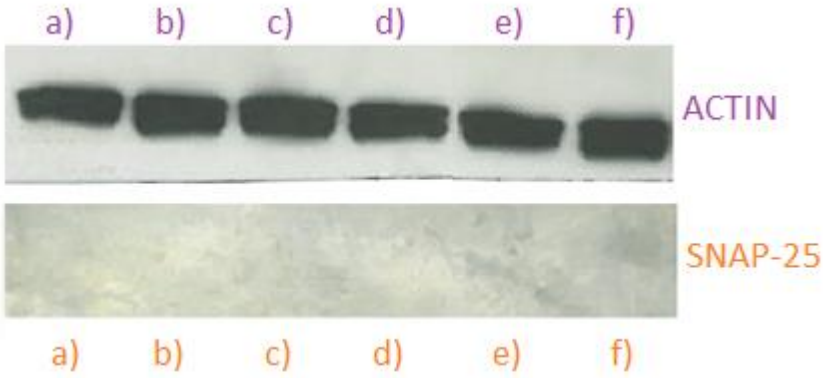


Figure 4.18: SNAP-25 blot analysis of undifferentiated BE(2)-C, SH-SY5Y and IMR-32 cells *(a) and (b) SNAP-25 BE(2)-C undifferentiated cells (a) and (b) Actin (c) and (d) SNAP-25 SH-SY5Y undifferentiated cells (c) and (d) Actin (e) and (f) IMR-32 undifferentiated (e) and (f) Actin. SNAP-25 detected at 25kDa and Beta-Actin at 42kDa.*

4.3.2.2.2 Expression of SNAP-25 by Mass Spectrometry Analysis

A high-density sample of SH-SY5Y cells was collected as described in the methodology and the protein concentration was analysed using a Direct Detect Concentrator (Merck Millipore). The total protein concentration was 0.755µg/µl; considerably lower than the BE(2)-C cell line concentration of 8.804µg/µl. Following the mass spectrometry sample preparation protocol, a second sample was collected to attempt an increase in protein concentration, with a resulting concentration of 1.94µg/µl. These low protein concentrations were similar to those obtained from the iCell neurons, discussed in Chapter 7, which suggested that SNAP-25 might not be detected easily in the SH-SY5Y samples. Limited availability of mass spectrometry analysis access initiated the decision to analyse only the BE(2)-C cells, IMR-32 cells, and the iCell neurons (refer to Chapter 7 for more detail).

4.3.2.3 IMR-32 Cell Line

4.3.2.3.1 Expression of SNAP-25 by Western Blot Analysis

Issues concerning the culture and differentiation of the IMR-32 cell line prevented successful analysis of differentiated cell expression of SNAP-25, but samples of undifferentiated IMR-32 cells were collected for Western Blot analysis. The blot shown in figure 4.15 indicates similar results for all the analysed neuroblastoma cell lines, and SNAP-25 was not successfully detected in the IMR-32 cell line in Western Blot in three repeated experiments.

4.3.2.3.2 Expression of SNAP-25 by Mass Spectrometry Analysis

Using a Direct Detect Concentrator (Merck Millipore) the protein concentration of the sample of IMR-32 cells collected for mass spectrometry analysis was analysed. The highest concentration sample results are shown in figure 4.19 and referenced in Chapter 7. The protein concentration for the presented sample was 11.824µg/µl; higher in comparison to the BE(2)-C, with a lower cell density collected in the sample (3 x 10^6 cells/sample).

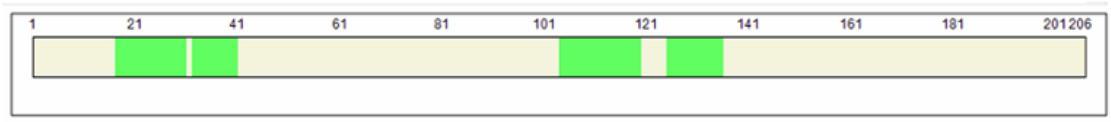


Figure 4.19: Mass Spectrometry SNAP-25 peptides detected in IMR-32 high cell density sample. *The peptides detected in the 206-amino acid protein are shown in green. 24% peptide coverage was detected.*

The mass spectrometry analysis showed that peptides corresponding to 24% of the whole SNAP-25 protein were detected (figure 4.19). The analysis of the IMR-32 cell line with mass spectrometry has demonstrated that SNAP-25 is expressed and detectable in a high cell density sample. The peptide coverage is significantly reduced in comparison to the BE(2)-C cells, which could be related to the fewer number of cells in the IMR-32 samples; the total protein concentration was however higher than the BE(2)-C cells.

4.3.3 iPSC-derived Cell Line SNAP-25 Expression Analysis

4.3.3.1 Expression of SNAP-25 in Human Neural Stem Cells

Collected protein samples were analysed by Western Blot for the detectable presence of SNAP-25 in the hNSCs cell line. Three replicates experiments were completed; collecting three separate samples containing approximately 775,000 cells and screened for the presence of SNAP-25. Each experiment revealed that SNAP-25 could not be detected in the hNSCs samples and a representative blot in shown in figure 4.20 below.

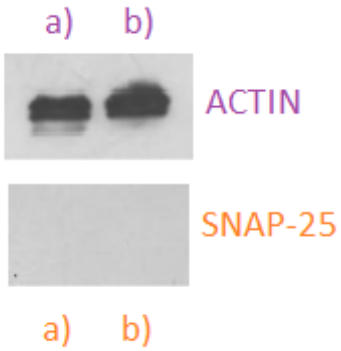


Figure 4.20: SNAP-25 Western Blot Analysis of Human Neural Stem Cells *(a) Actin toxin treated sample, discussed in chapter 5 (b) Actin untreated hNSCs sample (a) SNAP-25 toxin treated sample, discussed in chapter 5 (b) SNAP-25 untreated hNSCs sample. SNAP-25 detected at 25kDa and Beta-Actin at 42kDa.*

Whole SNAP-25 was not detected in the human neural stem cell samples, suggesting either low abundance or a potential error with the Western Blot analysis methodology. The latter is unlikely considering several experiments were conducted, each resulting in the same conclusion that the undifferentiated hNSCs do not express detectable levels of SNAP-25, if expressed at all. To confirm the results the hNSCs cell samples were run alongside the ReproCell ACH and DA samples, shown in figure 4.21 below, indicating that there were no antibody binding issues or errors within the Western Blot methodology.

As previously mentioned, the sample availability of the hNSCs was limited, so to maximise the potential analysis, toxin treated samples were also collected, and this is discussed in more detail in the proceeding chapter; Chapter 5; toxin sensitivity. The blots in the following figures also show the toxin treated analysis, which are not discussed at this point.

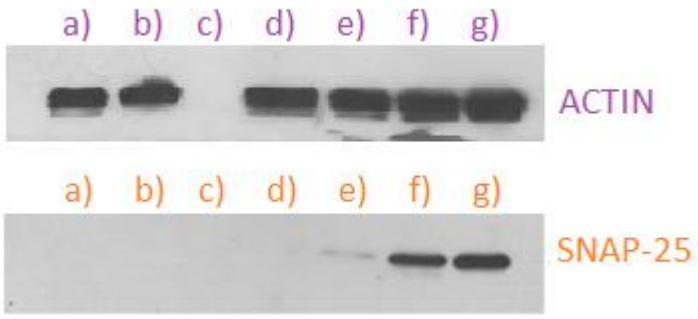


Figure 4.21: SNAP-25 Expression detected by Western Blot for hNSCs with ReproCell ACH and DA samples. *(a) SNAP-25 hNSCs (toxin treated) (a) Actin (b) SNAP-25 expression hNSCs (b) Actin (c) an unused lane (d) SNAP-25 DA (toxin treated) (d) Actin (e) SNAP-25 expression DA (e) Actin (f) SNAP-25 ACH (toxin treated) (f) Actin (g) SNAP-25 expression ACH (g) Actin. SNAP-25 detected at 25kDa and Beta-Actin at 42kDa.*

The toxin treated samples shown in both the Actin and SNAP-25 blots (a, d and f) are not the objective for discussion in this chapter and will be discussed in Chapter 5; toxin sensitivity. Many of the experiments were carried out using multiple samples on the same gel.

Figure 4.21 above shows the hNSC samples, alongside ReproCell samples, run on the same gel, in the same conditions indicating that there is not an issue with the detection of SNAP-25 in Reprocell samples. This suggests that the detection of SNAP-25 is working but that SNAP-25 may be in low abundance, if expressed at all, in the hNSCs cells.

4.3.3.2 ReproCell ACH and DA Cell Lines

The ACH and DA cell lines were analysed for the expression of SNAP-25 with Western Blot. The selected antibody for SNAP-25 is shown to be specific, and the blots produced clearly define both Actin and SNAP-25 in the ReproCell ACH and DA samples. The figure below shows two examples of the analysis and detection of SNAP-25 in both cell lines. With reference to figure 4.21 SNAP-25 is consistently detectable in the ACH sample, but visibly reduced in the DA, also shown in figure 4.22 below.

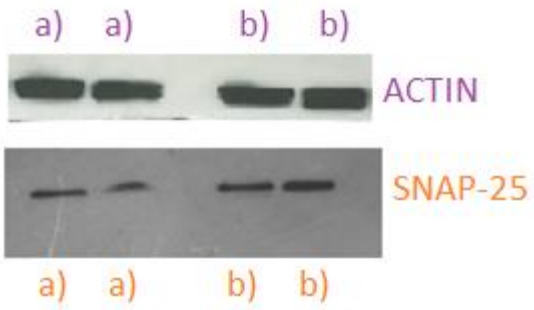


Figure 4.22: Western Blot Analysis of ReproCell Cell Lines *(a) SNAP-25 ReproCell DA (a) Actin ReproCell DA (b) SNAP-25 ReproCell ACH (b) Actin ReproCell ACH. SNAP-25 detected at 25kDa and Beta-Actin at 42kDa.*

A repeat of the experiments, provided a reduced number of viable cells on initial plating, (250,000 cells/mL ACH and 305,000 cells/mL DA cell line when cell density was supplied at 1 x 10_6 cells/mL for both ACH and DA) as discussed in Chapter 3. The results indicated that SNAP-25 expression is varying in the DA samples. Figure 4.23 shows a blot analysis of the samples, in which SNAP-25 is abundant in the ACH cells, but barely detectable in the DA.

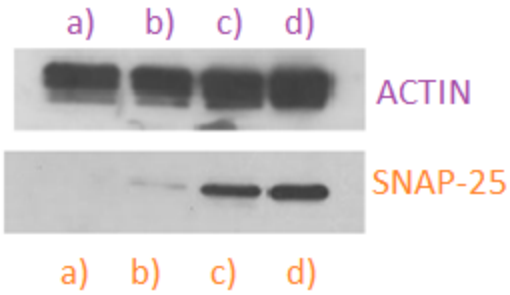


Figure 4.23: Western Blot Analysis of ReproCell ACH and DA cell lines. *(a) SNAP-25 DA (toxin treated) (a) Actin (b) SNAP-25 expression DA (b) Actin (c) SNAP-25 ACH (toxin treated) (c) Actin (d) SNAP-25 expression ACH (d) Actin. SNAP-25 detected at 25kDa and Beta-Actin at 42kDa.*

This variability was seen in each of the three samples collected from this vial, indicating that a strong variation in the number of viable cells contributes highly to the expression of SNAP-25 in the DA cell line when compared to the previous data. The ACH cell line expressed abundant SNAP-25, and appeared to be consistent in its expression; in both vials of cells and each experiment from each. This variance could be caused by the reduced number of cells, resulting in subsequent reduction in protein per sample, but the blot in figure indicates that SNAP-25 is not as present in DA as in the ACH cell line.

Three samples were analysed, but each was collected from the same vial of cells due to limited availability. This was the second vial of cells with a higher density on initial plating. The three replicates were quantified and the results are shown in figures 4.24 and 4.25.

The graphs show a more consistent expression of SNAP-25 in the ACH cell line in comparison to the DA cells. The amount of SNAP-25 expressed is marginally higher which is an interesting consideration in view of the number of cells; ACH being slightly less than the DA cell available cell number. This could suggest that the ACH cell line would be more sensitive than the DA in the use for a cell-based assay for Botulinum toxin type-A activity assessment and analysis. The p-values, calculated using a one-way ANOVA test for each cell line, indicate that there is

no significant difference between the amount of SNAP-25 expressed in each replicate experiment.

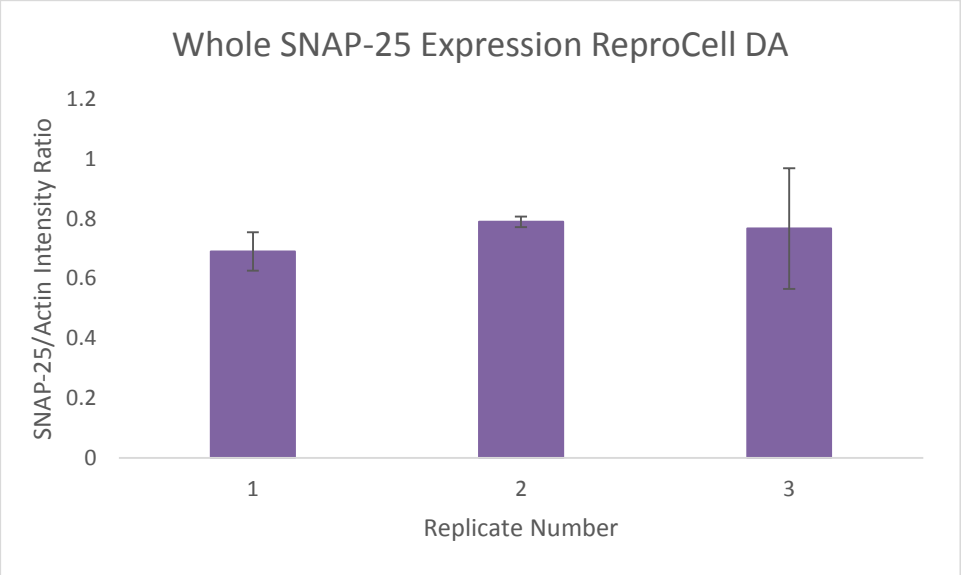


Figure 4.24: Bar chart representing DA ReproCell SNAP-25 expression analysis. *The bars represent the mean, of three replicate analyses (3 samples from the same vial of cells), uncleaved SNAP-25/Actin intensity ratio, and the error bars show the SEM of these figures. The results were repeated 3 times. N=3 p= 0.8147, one-way ANOVA.*

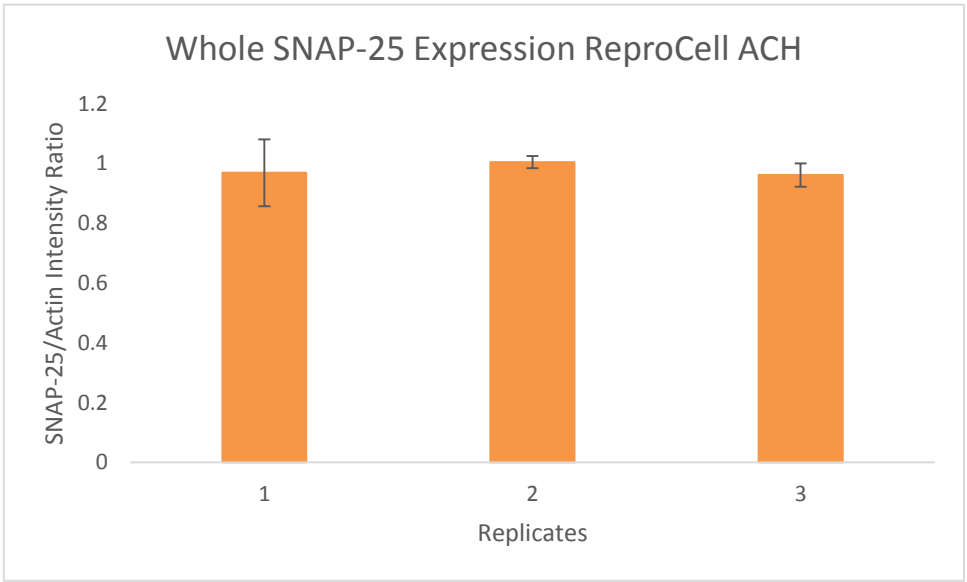


Figure 4.25: Bar chart representing ACH ReproCell SNAP-25 expression analysis. *The bars represent the mean, of three replicates, (3 samples*

from the same vial) uncleaved SNAP-25/Actin intensity ratio, and the error bars show the SEM of these figures. N=3, p= 0.7264, one-way ANOVA.

4.3.3.3 iCell Neurons

4.3.3.3.1 Western Blot

Collected iCell neuron samples were analysed by Western Blot for the detectable presence of SNAP-25 in the cell line. Three replicates experiments were completed; collecting three separate samples, on day 7 of culture, containing approximately 775,000 cells in 60µl of RIPA buffer and screened for the presence of SNAP-25. Each experiment revealed that SNAP-25 was successfully detected in the iCell neuron samples and a blot demonstrating two replicates is shown in figure 4.26 below.

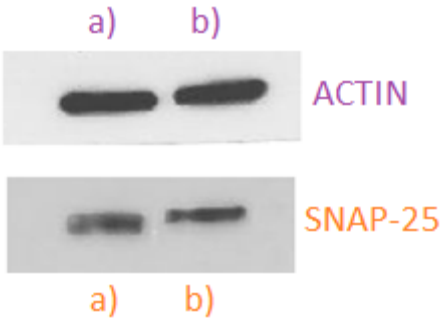


***Figure 4.26: Western Blot analysis of iCell Neuron cell line**. Example blot to show detection of SNAP-25, in two samples from different vials, collected on day 7. (a) and (b) SNAP-25 expressed (a) and (b) Actin. SNAP-25 detected at 25kDa and Beta-Actin at 42kDa.*

As defined in the introduction to this chapter, it is suggested that the iCells are viable for experimental use from 4-21 days. In order to evaluate the effect of growth time on the expression of SNAP-25 and to select the optimal treatment time, the growth of the cells was qualitatively assessed for 21 days (figure 4.27). Dendrite growth is visible from day 4 (figure 4.27a) and more complex neural networks are visible from day 7 (figure 4.27b) and continue to develop until day 21 (figure 4.27d). The cells are documented to be differentiated neurons, and if

the criteria by which the neuroblastoma cell lines were analysed are applied, the inhibition of cell proliferation and extension of neurite processes are clearly evident.

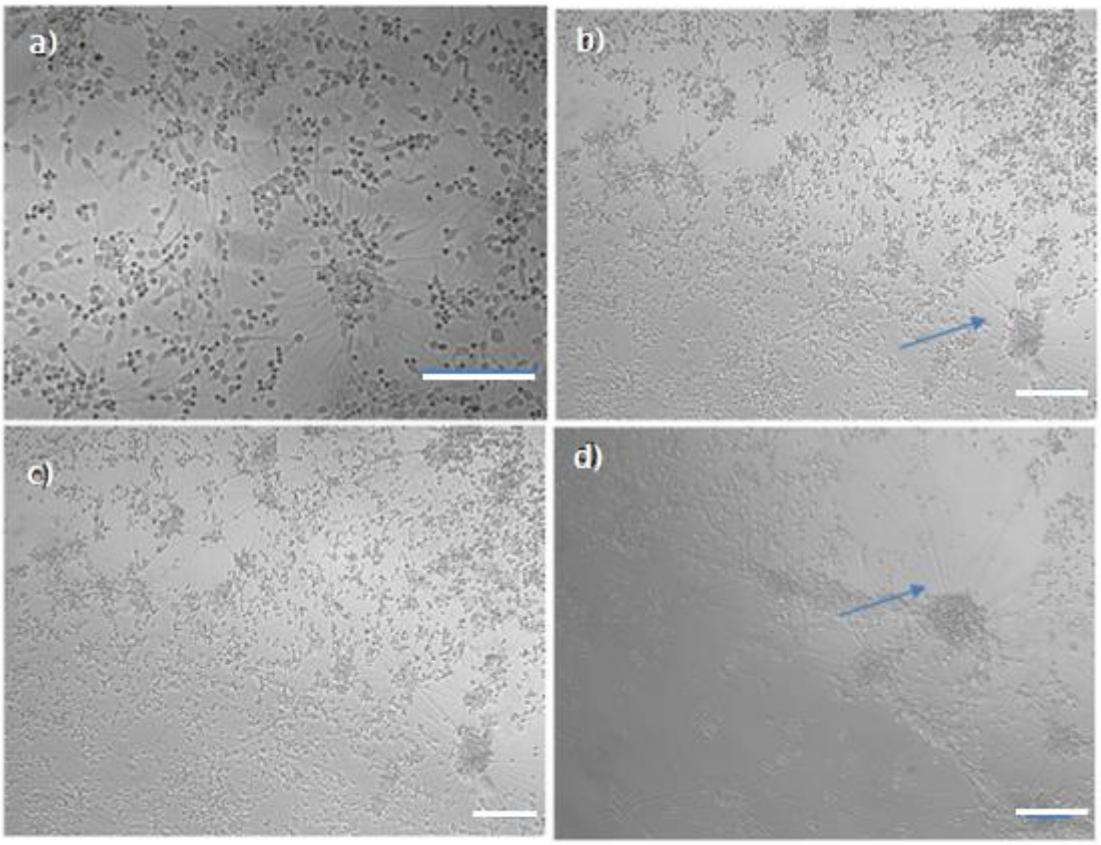


Figure 4.27: Growth of hiPSC-derived neurons. *These representative images illustrate the growth of cells over 21 days. a) day 4 b) day 7 c) day 14 d) day 21. Scale bar 100µm. Arrows indicate neurite formation and axon extension. Images were captured from one well of iCell neurons assessing growth over 21 days.*

The neuronal lineage of these cells is reflected in the morphology and neurite extension is prevalent by day 7, to analyse the variance in expression of SNAP-25 with relation to different culture times, samples were collected at 7 and 14 days to assess the expression levels of SNAP-25. An example blot comparing the expression of SNAP-25 at 7 and 14 days is shown below (figure 4.28).

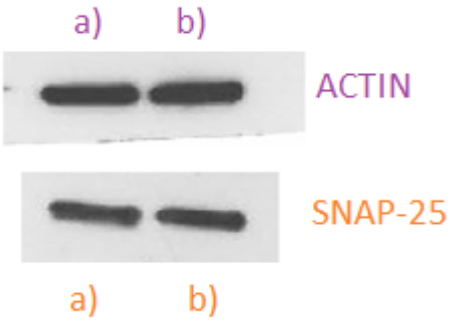


***Figure 4.28: Western Blot analysis of iCell Neuron cell line**. (a) SNAP-25 expressed at 7 days (a) Actin expressed at 7 days (b) SNAP-25 expressed at 14 days (b) Actin expressed at 14 days. SNAP-25 detected at 25kDa and Beta-Actin at 42kDa.*

Three samples were collected from each cell culture replicate, and as an indication of the insignificant difference in expression, the mean of 3 samples, for both 7 and 14 days are shown in the bar graph in figure 4.29. This data is from one replicate culture.

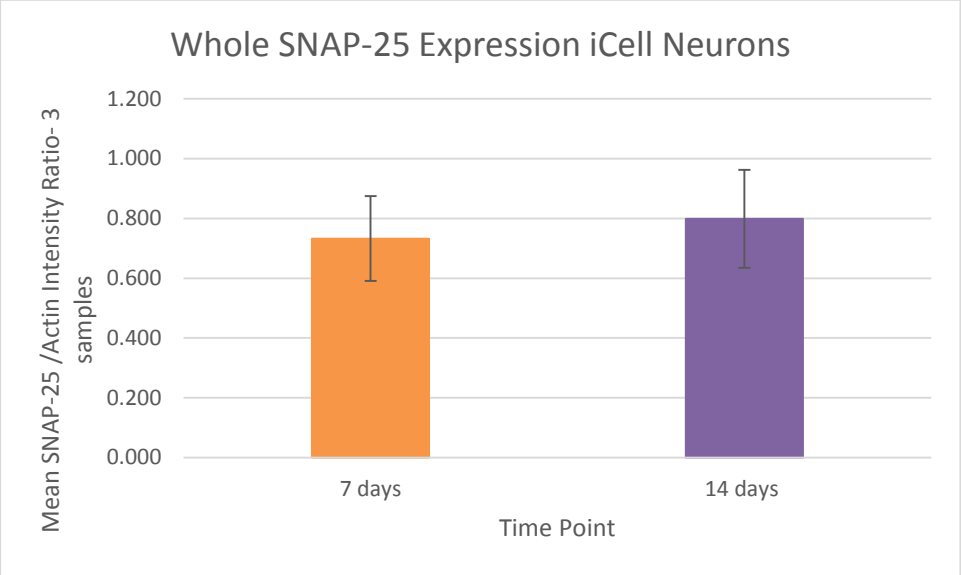


Figure 4.29: Bar chart representing iCell Neuron SNAP-25 expression analysis. *The bars represent the mean, of three replicate analyses (3 samples from the same vial of cells), uncleaved SNAP-25/Actin intensity ratio, and the error bars show the SEM of these figures. N=3 p= 0.7796, one-way ANOVA.*

The intensity of both Beta-actin and SNAP-25 were quantified using ImageJ and the SNAP-25 figure is normalised to Actin. This is the numerical figure expressed in the bar graph. The error bars show the standard error of the mean, and the difference between expression of SNAP-25 at 7 and 14 days, is statistically insignificant, demonstrated by the p-value of 0.7796.

In three replicate cultures, shown in figure 4.30 below, with three protein samples collected from each culture, there was also no meaningful difference in the levels of SNAP-25 expression between 7 and 14 days with a p-value of 0.1362. This suggests that the cells express maximum SNAP-25 for the cell type at 7 days, and growth time did not significantly increase the amount of SNAP-25 expressed (figure 4.30). The line graph below shows the three replicate cultures and the mean of SNAP-25 expressed for each of the three collected samples per culture. The intensity figure, used to calculate the mean result shown in the graph, is calculated by normalising intensity to the house-keeping protein Beta-Actin (SNAP-25/Actin).

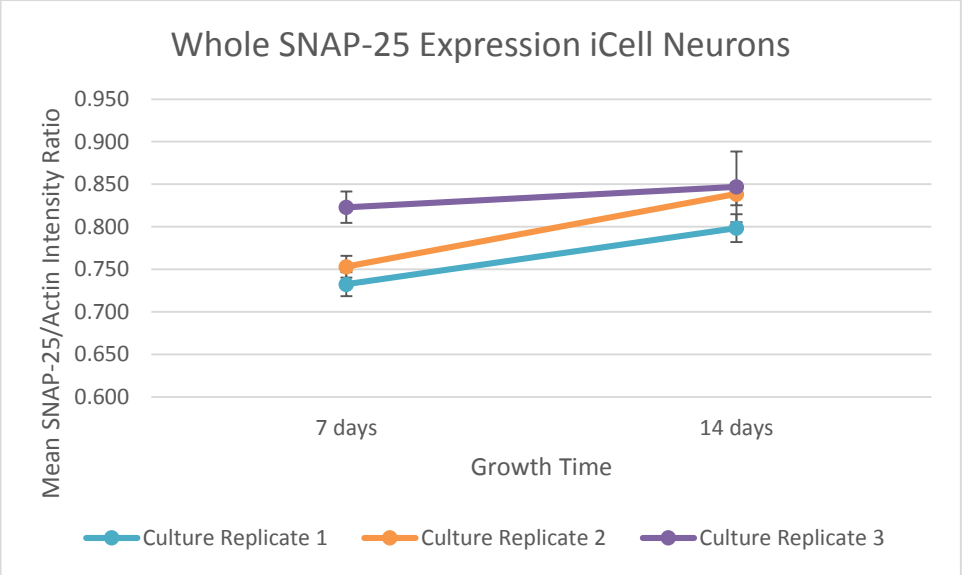


Figure 4.30: Line graph representing iCell Neuron SNAP-25 expression analysis. *The lines represent three replicate cultures, each with three sample analyses (3 samples from the same vial of cells), uncleaved SNAP-25/Actin intensity ratio, and the error bars show the SEM of these figures. N=3 p= 0.1362, one-way ANOVA.*

4.4 Discussion – Analysis of SNAP-25 Expression

4.4.1 Neuroblastoma Cell Lines

4.4.1.1 Neuroblastoma Differentiation

Two protocols were analysed for their potential to induce neuronal differentiation in the BE(2)-C, SH-SY5Y and IMR-32 cell lines; incubation in vasoactive intestinal peptide (VIP) and retinoic acid (RA). When assessing the differentiation protocols, it was imperative to apply the defined principles, in contribution to selecting the cell lines to proceed to criteria 3; toxin sensitivity. The principles that apply when analysing differentiation of cell lines, with regard to complying with a robust and repeatable method required for the cell-based assay, are summarised in the table below.

Differentiation Protocol Considerations and Requirements	
Cells differentiate easily, with a standardised procedure, and subsequent expression of SNAP-25 is improved.	Continue analysis of cell line- proceed to criteria 3
Cells express SNAP-25 without differentiation	
Cells do not respond to a differentiation protocol	Eliminate cell line as a possible candidate
Cells require a complex, variable protocol, with a mixed population of cells (that cannot be consistently replicated)	

Table 4.20: Defined protocol considerations and requirements in the assessment of VIP and RA differentiation. *Application of the criteria to each of the neuroblastoma cell lines.*

Application of these requirements to the results following differentiation with VIP and RA are discussed in the following sections.

4.4.1.1.1 Vasoactive Intestinal Peptide Differentiation

The VIP differentiation protocol did not yield conclusive results; the expression of Beta-III-tubulin in the BE(2)-C and SH-SY5Y cell lines was abundant, but as an early neuronal marker, it is suggestive that it would be present in immature neurons, which would need further differentiation to ensure complete differentiation to a neuronal cell type. The images shown for the beta-III-tubulin for both these neuroblastoma cell lines confirm the neuronal lineage of the cell lines, and the abundant beta-III-tubulin is clearly shown in the cytoplasm of the cells; confirming the staining is specific. The results for the BE(2)-C and SH-SY5Y cells in the ChAT staining, for an indication of the presence of cholinergic neurons were both negative; the VIP differentiation protocol does not generate cholinergic cell type in these neuroblastoma cell lines.

When scrutinising the MNR2 expression results for the BE(2)-C VIP differentiated cells, the presence of MNR2 could not be detected, even with the addition of undiluted primary antibody. Research shown in the Tanabe, et al, 1998 paper indicates MNR2 is expressed by motor neuron progenitors and expression is

sufficient to activate somatic motor neuron differentiation which can be identified by the expression of ChAT. This concludes that SHH-triggered differentiation is directed by the expression of MNR2 and that onset of MNR2 expression occurs when progenitors acquire individuality. This could suggest that the BE(2)-C cells would not be able to commit to motor neuron cell type, with the lack of expression of MNR2.

The fluorescent images captured for the SH-SY5Y VIP differentiated cells in the MNR2 expression analysis provided an indication that MNR2 is expressed in low abundance in some nuclei of the cells. This suggests that some of the cells within the VIP treated cell population have the capability to differentiate into motor neurons, and perhaps modifications such as an increased differentiation time or addition of growth factors could potentially increase the number of cells that express MNR2.

The IMR-32 cell line did not respond well to differentiation with both VIP and RA. The cells would detach from the plate within 2 hours of addition of VIP to the media, and high cell death was observed. The cells incubated in the control buffer, water, also detached, suggesting it is an issue with the cell line, as opposed to direct relation to the differentiation protocol. The observations surrounding this cell line, with reference to Chapter 3, is that there is an issue with the adherence of the cell line, reflected in standard cell maintenance procedures. The addition of differentiation reagents appears to amplify this issue and the immunocytochemistry analysis of the IMR-32 was rendered thus impossible.

The table below summarises the defined criteria in determining the success of the VIP differentiation protocol for each of the neuroblastoma cell lines. The BE(2)-C and SH-SY5Y cell lines, although more successful than the analysis of the IMR-32 cell line, still do not fulfil the entire criteria, and the immunocytochemistry results suggest that the VIP only induces the early stages of neuronal differentiation, with no cholinergic neurons present.

Criteria	BE(2)-C	SH-SY5Y	IMR-32
Extension of neurite processes	✓	✓	x
Increase in size of cells	x	x	x
Inhibition of cell proliferation	✓	✓	x

Table 4.21: Summary of the analysis of the neuroblastoma cell lines against the criteria for assessing the success of the differentiation with VIP.

These conclusive observations suggest that the VIP differentiation supports early neuronal differentiation for the BE(2)-C and SH-SY5Y cell lines, but when considering the defined principles, the variability of the results must be addressed. When reflecting on replicates of experiments, the BE(2)-C cells did demonstrate neurite extension and an inhibition of cell proliferation in all replicates, but never demonstrated an increase in the size of the cells. The immunocytochemistry results could only conclude that the cells were of neuronal cell type, with the detection of beta-III-tubulin, but the specific motor neurons and cholinergic neurons that could potentially be sensitive to botulinum toxin, were not present after 9 days of differentiation. The SH-SY5Y cell line also showed signs of differentiation and the immunocytochemistry reflected comparable beta-III-tubulin expression to the BE(2)-C cells and the expression of MNR2 was also detected in a select few of the cell population analysed. This highlights the variability in the cell type in the SH-SY5Y, potentially because of differentiation. If the cell line were to be selected for use in a cell-based assay, this variability would have to be monitored and controlled to ensure a consistent population with each test. The IMR-32 cell line could not apply any of the defined differentiation criteria, and the analysis suggests that the IMR-32 will be too variable if differentiation is required to ensure expression of SNAP-25, and subsequent potentially improved toxin sensitivity.

4.4.1.1.2 Retinoic Acid Differentiation

As mentioned in the results section, several attempts at RA differentiation were carried out for the BE(2)-C, SH-SY5Y and IMR-32 cell lines. After three attempts with a particular stock; which resulted in the cells detaching and dying after 48 hours or six days, the reagents used were replaced and the experiments were repeated.

Both the BE(2)-C and SH-SY5Y cell lines survived until the end of the experiments allowing the collection of samples for Western Blot analysis after 9 days of incubation in RA. The results of the blot analysis revealed that SNAP-25 is not detectable in the RA differentiated BE(2)-C or SH-SY5Y cells, with three replicate experiments concluding the same result.

When summarising the results from the analysis of the IMR-32 cells with the addition of RA, it can be concluded that this cell line will not be suitable for use, if differentiation is required to improve sensitivity to toxin. Addition of RA induced cell detachment within 72 hours, and a cell count provided an indication that the cells were dying easily as a result of exposure to RA and DMSO. With only 185 out of 3575 cells/µl being viable, it was decided to cease analysis of differentiated IMR-32 cells.

The Western Blot results for the analysis of expression of SNAP-25 in differentiated neuroblastoma cell lines concluded that SNAP-25 was not detectable, thus not improved as a result of differentiation. Three replicate cultures of RA differentiated BE(2)-C and SH-SY5Y cells were analysed; each concluding that SNAP-25 was not expressed at a detectable level in differentiated cells.

The table below summarises the defined criteria in determining the success of the RA differentiation protocol for each of the neuroblastoma cell lines. The BE(2)-C and SH-SY5Y cell lines, although more successful than the analysis of the IMR-32 cell line, still do not fulfil the entire criteria, and the western blot results indicated that there was no improvement in SNAP-25 expression or detection as a result of differentiation in RA. The results suggest that the RA on its own may only induce the early stages of neuronal differentiation, with no cholinergic neurons present.

Criteria	BE(2)-C	SH-SY5Y	IMR-32
Extension of neurite processes	x	x	x
Increase in size of cells	✓	x	x
Inhibition of cell proliferation	✓	✓	x

Table 4.22: Summary of the analysis of the neuroblastoma cell lines against the criteria for assessing the success of the differentiation with RA.

4.4.1.2 Summarising Neuroblastoma Differentiation

A consideration would be to extend the period of differentiation, and increase the potential for neuronal cell type, but the potential for a variable mixed population of cells, which cannot be regulated consistently, would be a likely outcome. An increase in the time to provide viable cells for testing leads to an increase of risk; variability in the cell population, and would suggest that it would be a complex and perhaps lengthy process.

The table below summarises the principles for differentiation when considering the cell line for continued analysis. The results are indicative of the observations from the VIP and RA differentiation protocols.

Differentiation Protocol Considerations and Requirements				
	BE(2)-C	SH-SY5Y	IMR-32	
Cells differentiate easily, with a standardised procedure, and subsequent expression of SNAP-25 is improved.	x	x	x	Continue analysis of cell line- proceed to criteria 3
Cells do not respond to a differentiation protocol	✓	✓	n/a	Eliminate cell line as a possible candidate BE(2)-C SH-SY5Y IMR-32
Cells require a complex, variable protocol, with a mixed population of cells (that cannot be consistently replicated)	✓	✓	n/a	

Table 4.23: Summary of the defined protocol considerations and requirements in the assessment of VIP and RA differentiation. *Application of the criteria to each of the neuroblastoma cell lines.*

4.4.1.3 Undifferentiated Neuroblastoma Analysis

The neuroblastoma were also analysed by Western blot for the presence of SNAP-25 in the undifferentiated cells. This would be an ideal component, with regard to easy of culture and preparation of the cells before testing in a routine laboratory environment, but the analysis conluded that SNAP-25 was not expressed at a detectable level by any of the neuroblastoma cell lines. This would suggest that the cell lines would need a complex differentiation protocol to encourage SNAP-25 expression for sensitivity to toxin which could result in an expression level that has the potential to change every time. This is perhaps too much of a risk and variability for approval from the FDA for a compliant assay for routine batch testing.

The analysis of each cell line was completed three times, as well as three times alongside the RA differentiated samples for the BE(2)-C and SH-SY5Y cell lines. Each experiment concluded that SNAP-25 was not detectable. The SNAP-25 standard control indicates that there is no issue with the method, and thus confirms that the level of SNAP-25 expression, is not detectable with the Western Blot method. Interestingly, BE(2)-C and IMR-32 high density cell samples were analysed as part of the mass spectrometry optimisation analysis (refer to Detection method Chapter for Mass Spectrometry results and section 4.3.2) and SNAP-25 was successfully detected in both cell lines. The peptide coverage of SNAP-25 for the BE(2)-C was significantly higher, at 54% than the IMR-32 cell line at 25%, but it was detectable, confirming that the cell lines do express SNAP-25, just not at an abundance detectable in low protein concentration cell samples by Western Blot. The number of cells used per sample to accumulate this detection of SNAP-25 would however not be feasible, or cost effective, for use in a cell-based assay.

The optimisation of the protocol for mass spectrometry analysis required several adjustments and attempts at analysis of the expression of SNAP-25, but the results from the BE(2)-C and IMR-32 cell lines show promise for both the methodology, and the use of the cell lines. Although SNAP-25 is expressed in the cells, the unsuccessful differentiation protocols and the highlighted complications that can occur as a result of a necessary requirement for differentiation for improved sensitivity suggest that the use of the neuroblastoma cell lines in a GMP-compliant cell-based assay would not be ideal or beneficial in terms of regulatory submission. Mass spectrometry is regarded as a far more sensitive detection method when compared to Western Blot, but the requirement of high cell numbers to ensure detection of SNAP-25 in the IMR-32 cell line is not indicative of criteria 1, defined in Chapter 3. To use a high number of cells to provide results could be problematic in the justification of its selection for use in a cell-based assay for testing botulinum toxin activity.

To summarise the observations of the neuroblastoma cell lines, and the expression of SNAP-25, each cell line is defined against the required principles specified when analysing the suitability of a cell line with reference to SNAP-25 expression. This is a key criteria in the determination of a cell lines suitability for use in a cell-based assay for the testing of BoNT-A. BE(2)-C is recorded in red, SH-SY5Y in purple and IMR-32 in orange (see table 4.24).

Principle/Standard	Cell Line Observations	Action
SNAP-25 Expression	SNAP-25 is expressed and detectable x x x	Consider cell line for criteria 3 analysis (toxin sensitivity)
	SNAP-25 cannot be detected following repeated experiments ✓ ✓ ✓	Eliminate cell line as a suitable candidate BE(2)-C, SH-SY5Y, IMR-32
Repeatability of SNAP-25 Expression	SNAP-25 is consistently expressed and detectable in repeated experiments x x x	Consider cell line for criteria 3 analysis (toxin sensitivity)
	SNAP-25 expression and detection is inconsistent in repeated experiments ✓ ✓ ✓	Eliminate cell line as a suitable candidate BE(2)-C, SH-SY5Y, IMR-32
Differentiation Protocols and Requirements	Cells express SNAP-25 without differentiation requirements x x x	Consider cell line for criteria 3 analysis (toxin sensitivity)
	Cells differentiate easily with a standardised procedure, and subsequent SNAP-25 expression is improved x x x	
	Cells do not respond to differentiation protocol ✓ ✓ ✓	Eliminate cell line as a suitable candidate BE(2)-C, SH-SY5Y, IMR-32
	Cells require a complex, variable protocol with a mixed population of cells (that cannot be consistently replicated) ✓ ✓ n/a	

Table 4.24: Principles and Requirements for Consideration for Criteria 2; Expression of SNAP-25. *The tabled criteria are considerations when analysing each of the selected cell lines for expression of SNAP-25 and the standards and requirements of the differentiation protocols. Observations for each neuroblastoma cell line are shown in colour-* ***BE(2)-C SH-SY5Y IMR-32****.*

4.4.2 iPSC-derived Cell Line Analysis

4.4.2.1 Human Neural Stem Cells

Section 4.3.3 indicates that the human neural stem cell line expresses either very low levels of SNAP-25, or no SNAP-25, with no detection with Western Blot analysis, with antibodies that have been confirmed to work. The slow growth, difficult maintenance and need for a further differentiation step to induce a more sensitive neuronal culture has suggested that this cell line would be susceptible to culture and cell type variance similarly to the neuroblastoma cell lines and indeed not suitable for use in this project. The expression of SNAP-25 is fundamental, and without expression suitability of the cell line can be ruled out. The first defined principle in the approach to criteria 2, expression of SNAP-25, is that this protein must be detectable in the cells. Without repeatable expression and detection, the cell line cannot be used for the testing of BoNT-A. The hNSCs could express SNAP-25, but in low abundance, and thus expression levels could potentially be increased by neuronal differentiation into a more sensitive cell type, but the slow proliferation discussed in Chapter 3 must be considered alongside the conclusion that SNAP-25 is not detected via Western Blot. The cell line chosen must be robust, repeatable in culture and reliable in testing approach; all of which are not indicative of or demonstrated by the hNSCs cell line.

This leads to the conclusive decision to thus rule out the human neural stem cell line based on its limited suitability for the cell-based assay.

4.4.2.2 ReproCell ACH and DA Cell Lines

The results analysing expression of SNAP-25 have suggested that a commercially differentiated cell line is more reliable in SNAP-25 detection and expression, and that the differentiation process appears to be a necessity in ensuring optimum expression levels of SNAP-25. However, although only two vials of each DA and ACH ReproCell cell lines were purchased, the variance in cell number and subsequent level of expressed SNAP-25 was much greater than two comparable vials of the iCell neurons.

SNAP-25 was successfully detected in both the ACH and DA ReproCell cell lines, and consistently expressed in each of the three samples collected from one vial.

There is slight variance in the amount of SNAP-25 expressed, in that it is more abundant in the ACH cell line compared to the DA, based on band intensity. This confirms the project hypothesis that cholinergic neurons express more SNAP-25 than dopmaninergic neurons, thus suggesting their potential sensitivity to toxin, with relation to the expression of SNAP-25. The variance appears when observing vial-to-vial cell density, and subsequent reduction in expression of SNAP-25 in relation to the reduced cell number. The second comparable vial did not allow for clear detection of SNAP-25 in the DA cells. The variance of expression of SNAP-25 is an issue in providing a robust and repeatable assay, and although the ACH cell line was consistent in its expression of SNAP-25, variability in the supplied number of differentiated cells would be an issue. As the ability to proliferate the cells is not feasible, the cell density on purchase needs to be consistent.

4.4.2.3 iCell Neurons

SNAP-25 was successfully detected in iCell neuron samples, at both 7 and 14 days and the specificity of the antibodies (refer to Appendix E) appear to be high and targets SNAP-25 with minimal background on Western Blot.

The cell density at initial plating has been extremely consistent, with no significant difference in the number of viable cells post-thawing and the supply of differentiated neurons is reliable, as discussed in Chapter 3. Thus, the expression of SNAP-25 was consistent with each collected sample, with detection of SNAP-25 at a cell density comparable to the hNSCs, which rendered no expression, and the ReproCell ACH and DA cell lines initial replicate experiments. The ease of detection of SNAP-25 in the iCell neurons lead to analysis of variance in expression of SNAP-25 in cells collected at 7 and 14 days. To have a 'window' of opportunity to use the cells at optimum conditions, without detrimental effect, would be extremely beneficial in a working environment, and analysis of two time points would highlight an increase, or decrease, in expression incoherent with time cultured.

The results indicate that there is minimal difference in SNAP-25 expression from 7 days to 14 days. This result is similar to the observations made in the Whitemarsh, et al, 2012 paper on which the experimental designs were based.

They looked at using the cells 4-14 days after plating and found that the cells yielded sensitivity to toxin at any time within these time points. Growing the cells for 7 days allows for adherence, dendrite extension and has been selected as the optimum time for growing iCells before exposure to botulinum toxin; this will allow for saving time when performing an assay for pharmaceutical potency testing, and the test would be relatively fast in set-up.

To summarise the observations of the iPSC-derived cell lines, and the expression of SNAP-25, each cell line is defined against the required principles specified when analysing the suitability of a cell line with reference to SNAP-25 expression (see table 4.25). These are key criteria in the determination of suitability for use in a cell-based assay for the testing of BoNT-A. Applied StemCell hNSCs is recorded in red, ReproCell ACH in purple, ReproCell DA in green and iCell neurons in orange (see table 4.25).

Principle/Standard	Cell Line Observations	Action
SNAP-25 Expression	SNAP-25 is expressed and detectable x ✓ ✓ ✓	Consider cell line for criteria 3 analysis (toxin sensitivity) ACH, DA, iCells
	SNAP-25 cannot be detected following repeated experiments ✓ x x x	Eliminate cell line as a suitable candidate hNSCs
Repeatability of SNAP-25 Expression	SNAP-25 is consistently expressed and detectable in repeated experiments x ✓ x ✓	Consider cell line for criteria 3 analysis (toxin sensitivity) ACH, iCells
	SNAP-25 expression and detection is inconsistent in repeated experiments ✓ x ✓ x	Eliminate cell line as a suitable candidate hNSCs, DA
Differentiation Protocols and Requirements	Cells express SNAP-25 without differentiation requirements x ✓ ✓ ✓	Consider cell line for criteria 3 analysis (toxin sensitivity) ACH, DA, iCells
	Cells differentiate easily with a standardised procedure, and subsequent SNAP-25 expression is improved x n/a n/a n/a	
	Cells do not respond to differentiation protocol n/a	Eliminate cell line as a suitable candidate n/a
	Cells require a complex, variable protocol with a mixed population of cells (that cannot be consistently replicated) n/a	

Table 4.25: Principles and Requirements for Consideration for Criteria 2; Expression of SNAP-25. *The tabled criteria are considerations when analysing each of the selected cell lines for expression of SNAP-25 and the standards and requirements of the differentiation protocols. Observations for each neuroblastoma cell line are shown in colour- **hNSCs, ACH, DA, iCells**.*

4.5 Conclusion – Analysis of SNAP-25 Expression

Based on the observations when applying the principles and standards required in the approach to selecting a sensitive cell line based on its expression of SNAP-25, several of the chosen cell lines can be eliminated following this analysis. SNAP-25 expression is crucial, and with consideration to the practicalities of using a cell line in a robust and repeatable experimental manner, the hNSCs cell line and neuroblastoma cell lines in particular are reviewed at this stage.

The IMR-32 cell line is eliminated based on its inability to survive or adhere to expected morphological characteristics in either of the differentiation reagents, and maintaining the cell line in culture is problematic. The expression of SNAP-25 was detected using mass spectrometry, but a high-density sample only provided 24% peptide coverage and detection, and an increase in the number of cells from approximately 3 x 10^6 cells per sample would not be an economically viable option for this projects use. The remaining neuroblastoma cell lines BE(2)-C and SH-SY5Y are also eliminated on the basis of SNAP-25 not being detected in Western Blot at a comparable cell density to the iCell neurons and ReproCell ACH and DA cell lines. SNAP-25 was detected with mass spectrometry in the BE(2)-C cell line but, like the IMR-32 cell line, the number of cells required in each sample to ensure detection of SNAP-25 is not an ideal characteristic.

The variability of the cell population in the neuroblastoma cell lines and the varying success of differentiation protocols has led to the suggestion, that a commercial cell line, purchased differentiated in a consistent population, would be a beneficial selection for a GMP compliant cell-based assay. This would reduce the variability of the cells used for potency testing, as Cellular Dynamics (iCell neurons) are able to provide a more consistent cell type within their product. The iCell neurons expressed consistent levels of SNAP-25, and considering the factors from Chapter 3 in terms of ease of culture and growth, the cell line is a promising candidate.

Although the cell density in ACH and DA ReproCell vials was drastically different, and the DA cells expressed varying and minimal levels of SNAP-25, the remaining cells, following collection for SNAP-25 expression analysis were treated with toxin as per Chapter 5, as they were available. If the toxin experiments

demonstrate a high sensitivity then the ReproCell cell lines will again be reviewed for use in the cell-based assay. The results are discussed in chapter 5.

Following the SNAP-25 expression experiments, the table below indicates the cell lines that are eliminated at this stage, and those selected for toxin analysis (criteria 3).

Cell Line		hNSCs	BE(2)-C	SH-SY5Y	IMR-32	ACH	DA	iCells
Criteria 1: Ease of Culture and Maintenance (Chapter 3)								
Proliferation		✓✓	✓✓✓	✓✓✓	✓	✓	✓	✓
Culture Maintenance		✓✓	✓✓	✓✓	✓✓✓	✓✓	✓✓	✓✓✓
Repeatability of Culture Conditions		✓	✓✓✓	✓✓✓	✓	✓	✓	✓✓✓
Criteria 2: Expression of SNAP-25 (Chapter 4)								
Expression of SNAP-25	**MS**	---	✓	---	✓	---	---	**x**
	WB	**x**	**x**	**x**	**x**	✓✓	✓	✓✓✓
Further analysis?		**No**	**No**	**No**	**No**	**Yes**	**Yes**	**Yes**

Table 4.26: Cell Line Conclusions Following Analysis Expression of SNAP-25. *Table outlines conclusive cell line observations and the selection of the cell lines for analysis of SNAP-25 expression in Chapter 4 and the conclusions from previous analysis in Chapter 3. One tick to three ticks progressively indicate the repeatability of SNAP-25 expression and of ease of culture; one is difficult to maintain or slow-growing and three ticks is very easy to maintain and high proliferation rate.*

Chapter 5: Toxin Sensitivity of Cell Lines

5.1 Introduction

Following the analysis of growth and culture and SNAP-25 expression (chapter 4) the neuroblastoma cell lines BE(2)-C, SH-SY5Y and IMR-32 and the human neural stem cells (hNSCs) have been eliminated as potential candidates for use in a cell-based assay. The conclusive results, at this stage, is that the expression of SNAP-25 is too variable, or non-existent, in the neuroblastoma and hNSC cell lines. Secondly, the variable detection of expressed SNAP-25 in the ReproCell ACH and DA reflected in Chapter 4 previously suggests that it is probable that this will exclude them as useable cell lines for the testing of Botulinum toxin type-A. However, toxin treated samples (30U/mL) were collected from the available cells and analysed; the results are discussed in the present chapter, with the variability demonstrated in chapter 4 being a primary consideration. The most promising cell line at this stage is the iCell neurons (Cellular Dynamics). With reference to ease of culture, the cells are purchased as differentiated GABAergic and glutamatergic neurons, so therefore do not require an extensive differentiation protocol before use. We have provided data that suggests that the cells can be used between 7 and 14 days, with no significant effect on the amount of SNAP-25 expressed (chapter 4) and have demonstrated consistent cell viability and cell number (Chapter 3, figure 3.14, pg 98) with reliable expression of SNAP-25. Criteria 3 of the approach to selecting a suitable cell line; sensitivity to toxin, is the next step in the cell line screening process. The remaining cell lines, following elimination of insensitive cell lines succeeding criteria 1 and 2, were analysed for the sensitivity to toxin in a series of experiments outlined in the present chapter.

Experiment principles are defined in table 5.1 below, which, if successfully analysed, will determine the cell lines sensitivity to toxin, forming a basis for the selection of the cell line for use in a cell-based assay. Following the determination of criteria 3, sensitivity to toxin, the cell line can be screened for receptor expression and proceed to the validation stage for GMP, ICH standards compliant use in a commercial setting. The sensitivity of a cell line to Botulinum toxin is of upmost importance as it is the defining basis of a functioning cell-

based assay for testing the toxin and determining its activity and subsequent potency.

	Principle/Standard	Cell Line Experiments	Action
1	Toxin Exposure- Incubation Time Comparison	6-hour incubation	Select optimal incubation time for further experiments
		48-hour incubation	
2	Toxin Uptake/Media Comparison	Stimulation media	Select optimal medium for future experiments
		Neuronal media	
3	Differentiation of Cleaved and Uncleaved SNAP-25	Both isoforms of SNAP-25 are detectable	Proceed with cell line- suitable selection
		Only uncleaved SNAP-25 is detectable	Optimise method or eliminate cell line as a suitable candidate
4	Repeatability of toxin treatment conditions	Cell line provides consistent and repeatable results.	Consider cell line for criteria 4 analysis (expression of receptors)
		Cell line is variable, results are inconclusive	Eliminate cell line as a suitable candidate

Table 5.1: Principles and Requirements for Consideration for Criteria 3; Toxin Sensitivity. *The tabled principles are considerations when analysing the toxin sensitivity of each of the remaining selected cell lines.*

5.1.1 Defining Criteria in the Analysis of Cell Line Sensitivity to Toxin

Principles defined as 1-3 in table 5.1 should be considered, in the approach to screening cell line sensitivity to toxin, in parallel and as primary aims. The differential detection of cleaved and uncleaved SNAP-25 (3) is compulsory for the analysis of toxin uptake and media comparison (2) and optimal toxin exposure relating to dose response (1). Repeatability of toxin treatment conditions (4) prominently applies, as with the other principles defined in previous chapters criteria, and the cell line should provide consistent results with each replicate experiment. This will define the cells lines suitability as a selected component of the cell-based assay in relation to repeatability and robustness of results with each experiment.

The experiments in this chapter analyse the cell lines ability to demonstrate sensitivity to toxin type-A, and allow the derivation of optimal protocols for the development of the cell line in use as part of a complete cell-based assay. The primary focusses are to detect and quantify differentiated cleaved and uncleaved SNAP-25 separately as a result of toxin exposure, which will provide confirmation that the toxin is able to enter the cells cytosol, translocate its light chain and cleave SNAP-25 sufficiently, and discovering optimum toxin treatment times through analysis of comparable incubation time and toxin uptake.

Toxin sensitivity of a cell line is defined by expression of SNAP-25 to allow for toxin activity and secondly that this activity can be detected. The process in which the toxin enters to cell and the light chain is translocated to subsequently cleave SNARE proteins; SNAP-25 in the case of Botulinum toxin type-A, is defined as toxin activity. A suitable cell line should express SNAP-25, as explored in Chapter 4 previously, and the cleavage of SNAP-25 should be detectable, ideally at a range of toxin concentrations.

The Western Blot method will be used for the initial analysis, but the selected cell line must also be compatible with the potential alternative in detection method. Previous results have demonstrated that the Western Blot is able to successfully detect SNAP-25 in the iCell neuron samples and is a viable method, at this stage, to provide preliminary results as to the cell lines sensitivity to Botulinum toxin type-A.

All experimental analyses were completed using Western Blot. SNAP-25 was successfully detected in cell samples, with a specific antibody providing repeatable results. At this stage of the project, Western Blot is the working and utilisable method for detection of SNAP-25 and was used to detect toxin activity via cleaved and uncleaved protein detection.

5.1.2 Dose Response and the Effect of Exposure Time

To define the cell line as sensitive to toxin experiments were designed to analyse optimal toxin incubation time for the accumulation of cleaved SNAP-25 and dose response to varying concentrations of toxin.

Experiments analysed comparable 6-hour and 48-hour toxin incubation, and sensitivity to varying concentrations of toxin at each incubation time-period. Literature suggests that the optimal toxin incubation time is 48-hours for the accumulation of cleaved SNAP-25 for detection (Whitemarsh, Strathman et al. 2012) but these comparative experiments will indicate whether the same results can be achieved in a shorter time frame.

5.1.3 Toxin Uptake and Media Comparison

Further experiments analysed toxin uptake in two comparable medias to determine whether addition of potassium chloride (KCl) and calcium chloride ($CaCl^{2)}$ to the media (referred to as stimulation media) can improve toxin sensitivity.

Synapses release NTs from synaptic vesicles that fuse with the plasma membrane to enable the release of their inner NT molecules. These molecules diffuse between the pre- and post-synaptic neuronal membrane and the synaptic vesicle is retrieved from the plasma membrane and turned into a new synaptic vesicle. This process is called synaptic vesicle recycling; a concept that BoNTs use to intoxicate neuronal cells (Rizzoli 2014)

Cells maintain a gradient between extracellular and intracellular calcium concentration and many membrane transport channels regulate calcium

homeostasis. Neurotransmitter release or synaptic transmission occurs when an action potential in the axon terminal causes the opening of voltage-gated calcium channels, increasing the internal calcium concentration of the axon terminal. This elevation is the signal that triggers the release of the neurotransmitter from the synaptic vesicles (Sudhof 2012). Membrane depolarisation stimulating the opening of Ca^{2+} channels and entry of extracellular Ca^{2+} into neurons drives synaptic vesicle recycling thus this would suggest that an increase in Ca^{2+} could encourage improved uptake of BoNT through synaptic vesicle recycling. Neurons utilise endocytosis to rapidly cycle synaptic vesicles on and off of the plasma membrane (Blum, Chen et al. 2012).

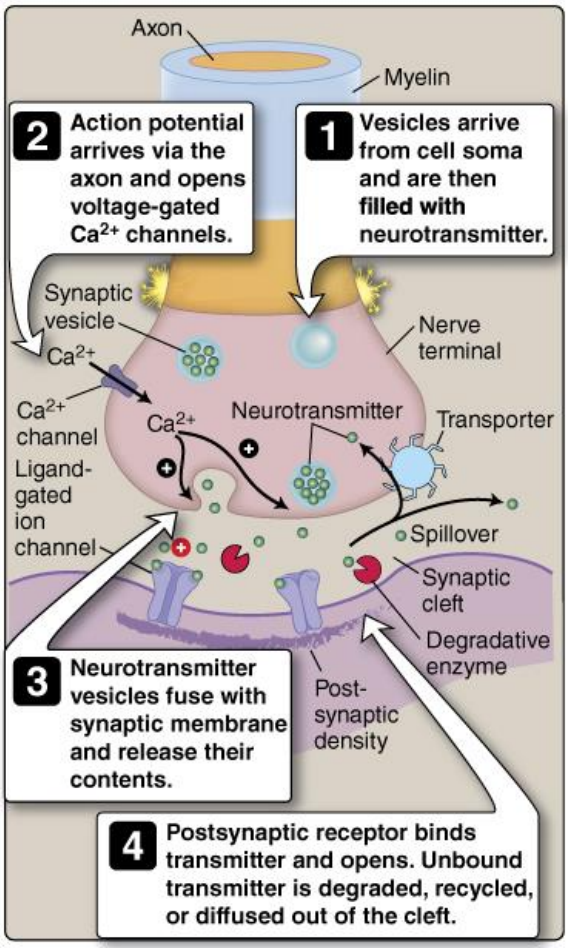


Figure 5.1: Diagram Representation of Neurotransmitter Release. *Release occurs when action potential enables the opening of calcium channels. Image taken from https://healtheappointments.com/chapter-5-nervous-system-organization-essays/2/ [online]. Accessed October 2017.*

A study by Keller (Keller, Cai et al. 2004) indicated that BoNT-A uptake in cultured neurons is enhanced with synaptic activity induced by potassium (K^+) depolarisation in the presence of Ca^{2+} and demonstrated that synaptic activity is essential for toxin endocytosis. During action potentials, potassium channels play an important role in the return of cells to a 'resting' state. To pair an increase in potassium with an increase in calcium should potentially stimulate neuronal synaptic activity and increase the rate of synaptic vesicle recycling.

To analyse toxin uptake and media conditions to ensure the optimal environment for toxin activity in the cells, a comparable experiment of dose times and media compositions was carried out. Activity dependence has been confirmed in literature, by exposing hiPSC-derived neurons to Botulinum toxin type-A for just minutes in stimulation medium; so, this methodology was optimised for the iCell neurons. It has been shown that significantly more SNAP-25 is cleaved in cells treated with stimulation media, at time points as short as 1 minute exposure to toxin; compared to the longer incubation required for a comparable result in the cells incubated in neuronal media (Whitemarsh, Strathman et al. 2012). Stimulation media is neuronal media with the addition of calcium chloride ($CaCl^2$) and potassium chloride (KCl), to concentrations of 2.2mM and 56mM respectively.

5.1.4 Botox vs Toxin

The botulinum toxin type A product used in these experiments differs in its preparation and diluent buffer compared to the commercially available Botox® product (Allergan Pharmaceuticals), a licensed botulinum toxin type A product used in the pharmaceutical and cosmetic industries. A comparative experiment, using the supplied toxin and commercial product toxin, in the same conditions ensures that the results are comparable to those published using Botox (Fernandez-Salas, Wang et al. 2012).

5.2 Methods – Toxin Sensitivity of Cell Lines

5.2.1 Dose Response and the Effect of Exposure Time- iCell neurons

Analysing the ideal toxin exposure time is fundamental to the experimental analysis of the remaining cell lines to ensure that the most productive methodologies are adapted for use in the complete cell-based assay. For example, if cells are incubated for a shorter amount of time, and the accumulation of detectable SNAP-25 is significantly increased with a 48-hour incubation time, this information is relevant to designing the best possible assay model. To analyse the optimum toxin incubation time for the detection of SNAP-25 two-time points were selected; 6 and 48-hours. Toxin concentrations were selected based on similar published results (Whitemarsh, Strathman et al. 2012), using lower concentrations for the 48-hour toxin incubation to prevent cell death in the initial experiments. The iCell neurons were plated on 96-well plates, with wells coated with 250µl/ well of 0.1% Poly-L-Ornithine at 37˚C for one hour and following a wash with 1x PBS pH7.4 coated in 8.3µg/cm² of Matrigel for thirty minutes (as previously described in chapter 3). The cells were plated at a density of 40,000 cells/well on 96-well plates. The concentrations for each time point; 6 or 48-hours, in table 5.3 below, were applied to analyse the effect of the toxin at different concentrations, in comparable incubation times. Both experimental designs incorporated a 0U/mL control, adding the toxin diluent to the media. After growing the cells for 7 days, toxin, or control buffer, was added to the media and the cells were incubated for either 6 or 48 hours. The toxin media was then removed, and disposed of in hypochlorite, and the cells were washed with 1x PBS 7.4pH three times to remove all traces of toxin. The cells were collected, as described in section 5.2.5, in RIPA buffer, collecting 15 wells (~600,000 cells) per sample concentration.

The toxin used in these experiments was provided at a potency of 7000U/mL. Stock dilutions of 1/10, 1/100 and 1/1000 were prepared for use in the dose response experiments; thus preventing the potential of using unrealistic quantities of toxin to portray the required U/mL concentrations for analysis. For example, large volumes of toxin thus preventing sufficient media volume in the wells to keep the cells viable, or volumes too small to measure toxin quantity accurately. Table 5.2 below shows the volumes of toxin and toxin diluent used to provide these stock solutions. Table 5.3 below shows the different amount of toxin and diluent used for each of the concentrations analysed.

Stock Dilution (Toxin 7000U/mL)	Volume Prepared	Toxin and Diluent Volumes
1/10	300µL	30µL toxin
		270µL diluent
1/100	800µL	80µL 1/10 stock toxin
		720µL diluent
1/1000	100µL	10µL 1/100 stock toxin
		90µL diluent

Table 5.2: Toxin Stock Diluted Preparations for use in Dose Response Experiments.

Incubation Time	Toxin Concentration	Volume/100µL 96-well plates	
		Toxin	Diluent
6-hour	1000U/mL	14.3µL	85.7µL
	500U/mL	7.15µL	92.85µL
	100U/mL	14.3µL of 1/10 stock	85.7µL
	0U/mL	14.3µL (highest volume)	85.7µL
48-hour	30U/mL	42.8µL of 1/100 stock	57.2µL
	10U/mL	14.3µL of 1/100 stock	85.7µL
	0.1U/mL	1.43µL of 1/1000 stock	98.57µL
	0U/mL	42.8µL (highest volume)	57.2µL

Table 5.3: Toxin Concentrations for Exposure Time Analysis Experiments. *All toxin was from the same batch for each experiment. The diluent is provided with the toxin and used in the control for each experiment. The volume of buffer for the 0U/mL control equates to the maximum volume of toxin added to the media.*

The diluent used in the control samples is used in the production of the botulinum toxin drug product used in these experiments. The toxin and diluent are provided by a client of Wickham Laboratories and the components of the diluent are confidential.

5.2.2 Toxin Uptake/Media Comparison- iCell neurons

These experiments look at comparative exposure times of the cells to toxin to assess how quickly the toxin is taken into the cell to target SNAP-25, in two different media types; neuronal and stimulation media. Neuronal media is the maintenance media provided with the iCells, with the addition of B27 (Thermofisher #17504001), Glutamax (Thermofisher #35050061) and antibiotics to prevent contaminations (see table 5.4). Stimulation media is composed of neuronal media with the addition of $CaCl_2$ (calcium chloride) and KCl (potassium chloride) to make the final concentration of 2.2mM and 56mM respectively (see table 5.4). iCells were plated on 96-well plates, coated as described in section 5.2.1 at 40,000cells/well on 96-well plates, and grown for 7 days before starting the experiment. Toxin was added to each media at a concentration of 550U/mL and added to the cells for 1, 5 or 10 minutes, then toxin media was removed from each well. Wells were washed with maintenance media, which is the iCell basal media without the additional reagents, three times to remove all traces of toxin, then filled with fresh maintenance media (100µL/well) and incubated at 37˚C for 24-hours, before collection of the cells. Due to the cost limitations of the cells, 8 wells per concentration were collected, 320,000 cells/sample concentration (3 replicate experiments), in 40µL of RIPA buffer.

Media	Composition for 5mL	Exposure Time (550U/mL toxin)
Neuronal Media	iCell maintenance media – 4.35mL	0 (no toxin)
	2% B27 - 100µL	1 minute
	2mM Glutamax - 50µL	5 minutes
	Penicillin/Streptomycin - 500µL	10 minutes
Stimulation Media	Neuronal media (as above)	0 (no toxin)
	2.2mM CaCl2	1 minute
	56mM KCl	5 minutes
		10 minutes

Table 5.4: Media Composition of Neuronal and Stimulation Media. *Table of comparative media composition and toxin uptake time experimental plan. The 0-minute no-toxin controls incorporated toxin buffer (diluent) incubation for 10 minutes.*

5.2.3 Assessing the Activity of the Toxin

As previously described, the toxin used in this project differs from the commercially available Botox® product in relation to diluent and experiments were designed to compare the activity of both products to ensure toxin consistency and compare efficiency. Plates were prepared with 0.1% Poly-L-Ornithine and Matrigel coating, as described previously in section 5.2.1, and the iCell neurons were plated at 225,000cells/well on 24-well plates. The Botox® drug product was diluted to a 30U/mL stock solution with saline, as per the manufacturers instruction, and then added to the media in each well (table 5.5). The toxin was also diluted to 30U/mL and added to the cells. The control experiments analysed Saline, the Botox diluent, and Buffer, the toxin drug product diluent used in previous experiments and which has already been shown to have no negative impact on cell survival. The equivalent volume of buffer: toxin was added (30µL toxin added for Botox experiment, 30µL of saline added to control). Each sample was collected in 60µL of RIPA lysis buffer (3 wells of 225,000 cell/well) after 48 hours of toxin treatment for Western-blot analysis.

Solution	Concentration	Volumes
Botox 30U/mL	Botox 100U stock diluted to 15U/500µl	Botox- 50µl/well Media- 450µl/well
Saline 0U/mL	Undiluted	Saline- 50µl/well Media- 450µl/well
Toxin 30U/mL	Toxin 500U stock diluted to 15U/500µl	Toxin- 30µl/well Media- 470µl/well
Buffer 0U/mL	Undiluted	Toxin Buffer- 30µl/well Media- 470µl/well

Table 5.5: Botox and Toxin Concentrations for Comparing Toxin Activity. *The Botox was reconstituted to a 100U stock, as per the manufacturer's protocol, and subsequently diluted to 30U/mL for testing. The toxin was also diluted to a comparable 30U/mL*

5.2.4 ReproCell ACH and DA Toxin Experiments

Although there are reservations concerning the reliability of the expression of SNAP-25 with both the ACH and DA cell lines suggested from results in chapter 4, toxin sensitivity analysis was carried out on the cells. The limited cell number restricted the availability of cells for toxin analysis; the cells do not proliferate, so one control and one toxin treated sample per cell line were collected (see table 5.7). Plates were coated in 0.002% Poly-L-Orthinine (24-well) and the provided coating solution (table 5.6) before the cells were plated at a density of 225,000 cells/well. The ACH and DA cell lines were cultured for 19 days, as per the recommended protocol, and media changes were carried out every 2-3 days, using maturation media, different for ACH and DA cell lines (table 5.6). Toxin was diluted in maturation media to a concentration of 30U/mL, and added to the cells (see table 5.7). The cells were incubated in the toxin media at 37˚C for 48 hours, then washed three times in maturation media before being collected to remove all traces of toxin. The cells were collected in 60µl RIPA buffer; 3 wells

per concentration, as described in section 5.2.5 below and stored at -80˚C awaiting Western Blot analysis.

Solution	Components	Concentration (prep 40mL)
PLO Coating Solution	Poly-L-Orthinine 0.1%	0.002% in PBS
Coating Solution	Provided by Supplier- no dilution	
Thawing Media	Provided by Supplier- no dilution	
ACH Maturation Media	Maintenance Media Additive 'A' Penicillin/Streptomycin	38.88mL 720µL 400µL
DA Maturation Media	Maintenance Media Additive 'A' Penicillin/Streptomycin	39.08mL 520µL 400µL

Table 5.6: Solutions required for the preparation of the ReproCell ACH and DA cell cultures for toxin sensitivity experiments.

Incubation Time/Cell Line	Toxin Concentration	Volume per well
48-hours ReproCell ACH	30U/mL	30µl toxin 470µl media
	0U/mL	30µl toxin diluent 470µl media
48-hours ReproCell DA	30U/mL	30µl toxin 470µl media
	0U/mL	30µl toxin diluent 470µl media

Table 5.7: Toxin Concentrations for ReproCell ACH and DA cell lines experiments.

5.2.5 Collecting Cell Samples

The toxin sensitivity experiments were initially on 96 well plates, and 15 wells per sample collected in 60µL of RIPA buffer with protease inhibitors (see table 5.8). Developing experiments lead to the change to 24 well plates, ensuring that 3 wells were collected per sample in the same volume of buffer (approximately the same number of cells per sample). The cell collection protocol was carried out on ice to prevent protein degradation. Cells were collected by pipetting buffer into each well then proceeding to scratch a pipette tip along the base of each well, ensuring all cells are removed from the plate and transferred to a cold tube in the RIPA buffer. The solution is centrifuged at 4˚C at 300 xg for 15 minutes; this breaks down cell walls and release proteins into the buffer, separating protein lysate from cell debris. The supernatant is transferred to a new tube and stored at -80˚C before Western Blot analysis.

Solution	Components	Concentration
RIPA Buffer	NaCl	150mM
	NP40	1%
	TRIS pH 7.5	50mM
	EDTA	1.5mM
	Sodium Deoxycholate	1g/100mL
	SDS	0.1g/100mL
Protease Inhibitor (Cocktail Set III- Calbiochem Merck #539134)		1:100

Table 5.8: RIPA Buffer Components.

5.2.6 Initial Protocol for Western Blot Analysis

Cell lysates were analysed by Western Blot to quantify the expression of both cleaved and uncleaved SNAP-25 demonstrating toxin activity. This detection method was used in the screening process as it provides an indication of SNAP-25 cleavage using an anti-SNAP-25 antibody, detecting both cleaved and uncleaved SNAP-25 protein bands bound on a membrane; allowing quantification of cleavage and thus determining toxin activity.

Samples were mixed 1:2 in LDS sample buffer (Life Technologies). A protein concentration analysis, using a BSA protein analysis kit, of collected iCell neuron samples indicated low abundance of protein. It was decided that it was best to eliminate a protein concentration analysis with each replicate experiment to prevent the use of limited sample before each experiment. The cell number was carefully plated each time to try and ensure consistency and 25µl of each sample was loaded onto on 12% TGX Protean gels, with 1 well (10µl/well) of BioRad Dual Colour Markers; a molecular marker ladder for identifying protein bands (see Appendix C). The Western Blot migration and transfer protocol was carried out, as described in chapter 4, section 4.2.1 and Appendix D.

The blots were incubated overnight in the primary rabbit polyclonal SNAP-25 antibody (Synaptic Systems #111 002) at a concentration of 1:1000 (appendix A) or Beta-actin rabbit polyclonal antibody 1:5000 and then washed in 1x TBS (refer to Appendix E for full list of antibodies used). The blots were incubated in HRP-anti-rabbit for 1 hour then protein bands revealed on film with enhanced chemiluminescence (ECL) (see Appendix G for protocol). Quantification of the protein bands was performed using ImageJ software (Appendix H).

5.2.7 Optimised Western Blot Protocol

The preliminary results shown in section 5.3 demonstrate only uncleaved SNAP-25 detection. As defined in the introduction to this chapter, the detection of both cleaved and uncleaved SNAP-25 is essential for the quantification of toxin activity, therefore the methodology for detection of SNAP-25 required optimisation. The gels used for the Western Blot analysis were changed from 12% TGX gels to 4-20% gradient gels to provide a greater separation of bands at

the base of the gel. SNAP-25, when cleaved as a result of toxin activity, can be detected at 24kDa, which is the cleaved portion of SNAP-25, with the remaining uncleaved SNAP-25 being detected at 25kDa. These molecular weights are obviously close together and difficult to separate and distinguish between in a Western Blot. With the adaptation of the methodology, we were able to detect the cleaved band at 24kDa, separately from the uncleaved at 25kDa. Cooling the running and transfer buffers and increasing the time for protein migration through the gel would separate the bands further, so the protocol was altered to run the gel until the 15kD marker was at the base of the gel. These slight alterations lead to the detection of the cleaved band, as shown in figure 5.11 in the results section of this chapter. Samples were prepared as described in section 5.2.6, using LDS sample buffer and heating to 95˚C before adding the samples to the gel. The altered methodology incorporates an extended migration time of 30 minutes to 50 minutes to ensure separation of cleaved and uncleaved SNAP-25. Quantification of the detected bands was calculated using ImageJ software.

5.3 Results – Toxin Sensitivity of Cell Lines

5.3.1 Dose Response and the Effect of Exposure Time- iCell Neurons

Detecting the effect of various concentrations of toxin determines the sensitivity of the cell line and a comparison of exposure times, 6 hours and 48 hours, suggests the optimum incubation time for detection of cleavage of SNAP-25. The results for the preliminary experiments investigating cell toxin sensitivity and exposure times follow.

Figure 5.1 below shows one representative Western blot analysis of the iCell neuron samples collected for the 6 and 48-hour experiments.

Figure 5.2: Western Blot of Dose Response and Exposure Time Analysis. *The blot shows one representative experiment. iCell neurons incubated in different concentrations for 6 or 48-hours. Actin bands labelled in purple, SNAP-25 labelled in orange.* ***(a)*** *1000UmL 6-hours* ***(b)*** *500U/mL 6-hours* ***(c)*** *100U/mL 6-hours* ***(d)*** *0U control 6-hours* ***(e)*** *30U/mL 48-hours* ***(f)*** *10U/mL 48-hours* ***(g)*** *0.1U/mL 48-hours* ***(h)*** *0U control 48-hours.*

The blot shown above demonstrates detection of uncleaved SNAP-25, with no cleaved SNAP-25 band visible. The results below are calculated from the mean of three separate experiments, each only revealing the uncleaved SNAP-25 band for quantification.

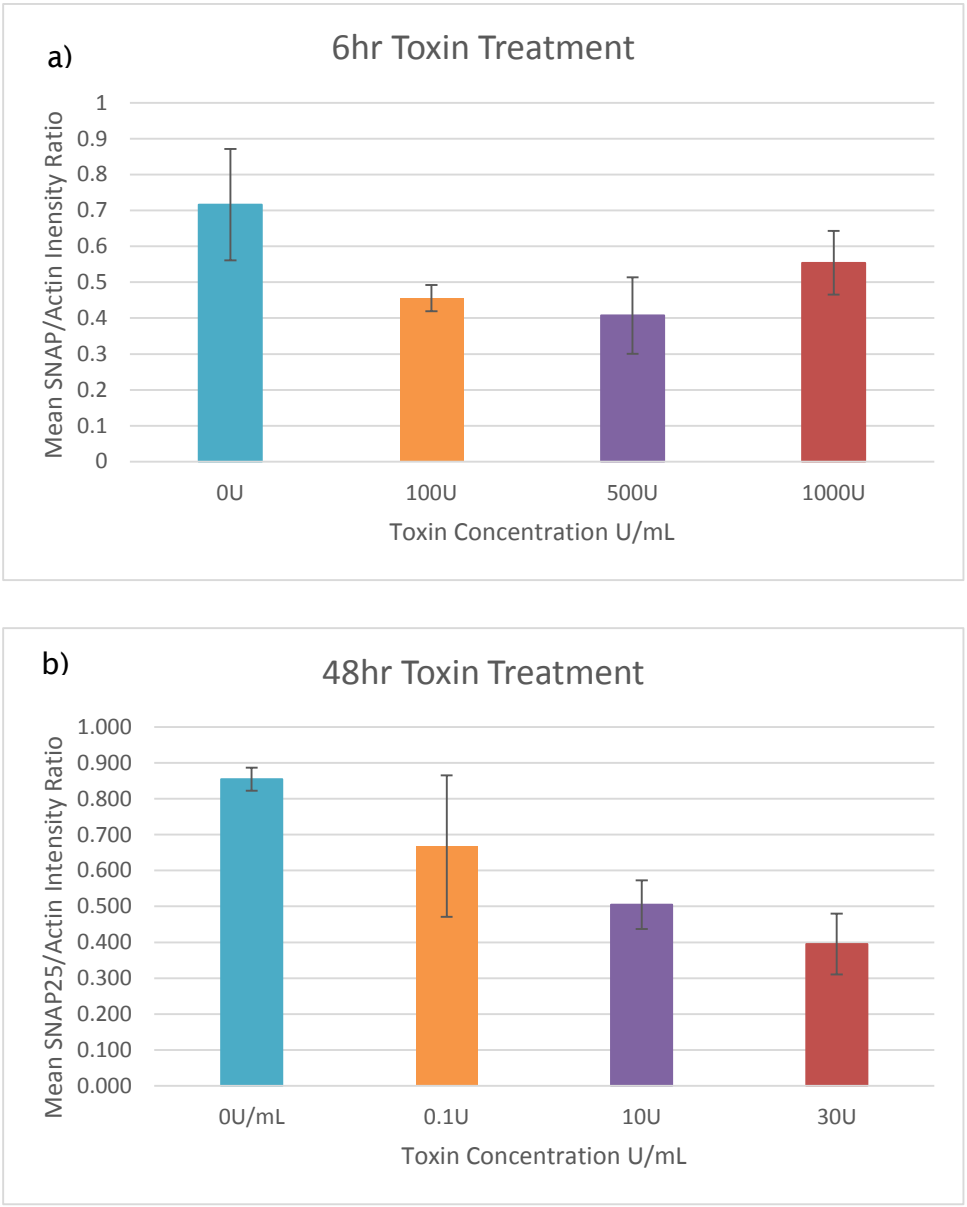


Figure 5.3: Comparison of BoNT-A Incubation Times. *6 hours (figure 5.2a) and 48 hours (figure 5.2b) toxin treatment on iCell neurons. The mean of the uncleaved SNAP-25 to Beta-Actin intensity ratio, of three replicates, is reflected in the bar chart. The error bars indicate the SEM. Detection of whole SNAP-25 only, no cleaved band detection. 6-hour experiment p=0.4883, no significant difference between toxin concentrations. 48-hour experiment p=0.1172, no significant difference between toxin concentrations. Statistical analysis using one-way ANOVA method.*

The graphs shown in figure 5.3 compare the efficiency of toxin activity when cells are exposed for 6 or 48-hours. Figure 5.3a shows the results of the 6-hour toxin exposure, indicating that there is a 43% cleavage of SNAP-25 (57% uncleaved detected), when compared to the 0U control band intensity for the 500U/mL sample. According to the data shown in this graph, which is the mean of three replicate experiments, the 500U/mL concentration of toxin is the most efficient in the restricted 6-hour exposure of the cells to toxin. The 1000U/mL sample only rendered an average of 23% cleavage, and the 100U/mL 36% cleavage, based on the detection of only the uncleaved portion of SNAP-25.

Figure 5.3b shows the data collaborated from three replicate experiments for the 48-hour exposure time. The most positive result was obtained using the 30U/mL toxin concentration, which rendered 54% SNAP-25 cleavage; based on detection of the remaining uncleaved protein only. 10U/mL provided 41% cleavage, but 0.01U only 20%. The standard error of the mean for the 0.01U experiments is very wide suggesting that this result could perhaps not be as reliable as the other data in these experiments.

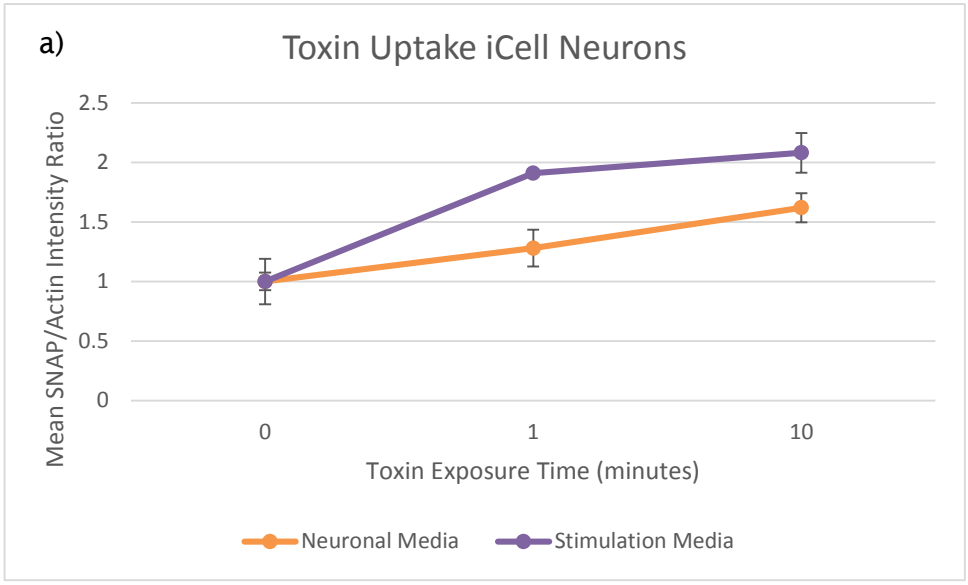
Based on these results, the optimum concentration for screening cell line suitablilty for use in a routine cell-based assay is incubation with 30U/mL toxin for 48-hours; yeilding a 54% cleavage of SNAP-25.

The statistical analysis, using a one-way ANOVA test, indicated that there is no sigificant difference between the toxin concentrations used in the 6-hour toxin incubation experiments, with a p-value of 0.4883. The cleavage of SNAP-25 is apparent in the detection of a reduced amount of uncleaved SNAP-25, in comparison to the control, for each of the toxin concentrations but is not significantly improved with an increase in toxin concentrations. This is a similar result as shown by the statistical analysis of the 48-hour toxin incubation experimental data. The one-way ANOVA indicated that there was no significant difference in the amount of SNAP-25 cleavage across the concentrations of 0.1U, 10U and 30U. The percentage of SNAP-25 cleaved demonstrated by the 30U toxin analysis was the highest, though not statistically significant in comparison to the 0.1U and 10U experiments, and was therefore selected as the optimum concentration for the screening of cell line suitability.

5.3.2 Toxin Activity and Media Comparison

The paper by Whitemarsh (et al, 2012) indicated that when cultured in stimulation media, the cells showed improved sensitivity to toxin when exposed for 5, 10 or 15 minutes in comparison to neuronal media. We replicated this experiment with different time points to test the effect of media environment on the synaptic activity of the cells.

Figure 5.4 below appears to show a more defined increase in toxin activity and uptake, with the resulting SNAP-25 cleavage, for the stimulation media but the two-way ANOVA analysis of the data suggests no statistical difference in the two data sets. The results demonstrate that in the neuronal media approximately the same amount of SNAP-25 is cleaved when exposed for 1 minute or 10 minutes. The difference in amount cleaved is minimal over the two time points. The stimulation media however demonstrates a clear increase in the SNAP-25 cleaved, comparing the one-minute exposure to the result of 10 minutes. At all time points the amount of SNAP-25 cleaved is greater in stimulation media than in the neuronal media, suggesting the involvement of synaptic activity in the mechanism of action of the toxin based on detection of uncleaved SNAP-25 compared to the 0U control.



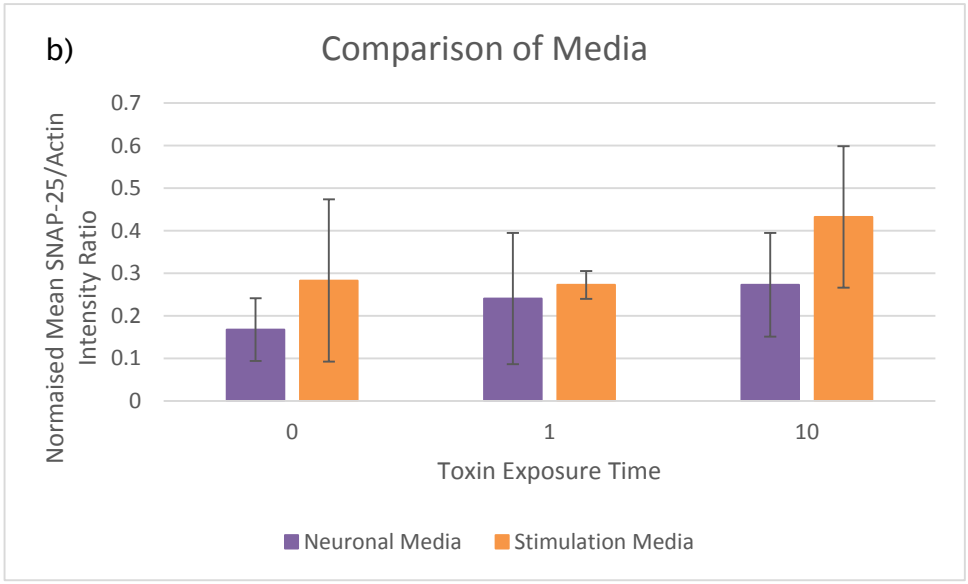


***Figure 5.4: Toxin Uptake Comparison**. **(a)** Line graph displaying the mean ratio of two data replicates normalised to the 0U figure **(b)** Bar chart displaying mean SNAP-25/actin intensity ration, error bars represent SEM n=2. Two-way ANOVA analysis provided a p-value of 0.653, confirming statistical insignificance.*

A statistical comparison of the two incubation times in each of the tested medias suggested that there was no statistical difference or improvement with either media. The calculated p-values show not enough variation in the data to be significant and suggest either media is more efficient in encouraging toxin activity. The cost and limitations of the cell line limited the number of experiments carried out, without successful detection of both cleaved and uncleaved SNAP-25, so only 2 replicate experiments were completed, suggesting that the statistical analysis is only a suggestion of insignificance across data sets. This would need to be established with n=3 to confirm.

5.3.3 Toxin Efficiency Comparison; Botox® and Supplied Toxin

As previously mentioned, the detection of both cleaved and uncleaved SNAP-25 and the distinction between the two isoforms had proven problematic. The efficiency of the toxin was a cause for consideration and its possible effect on lack of detection was scrutinised. An analysis of the efficiency of the toxin used was carried out, to ensure that comparable results are achieved when using the commercially available alternative toxin product Botox®. This would demonstrate if the toxin works as it should and highlight any possibilities of degradation of SNAP-25 due to the chemical make-up of the toxin and diluent used. The results from a Western Blot analysis are shown in the figure below.

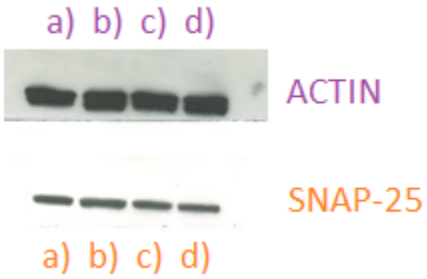


Figure 5.5: Western Blot Analysis of Botox® and Supplied Toxin. *SNAP-25 expression; only uncleaved SNAP-25 detected. Actin labelled in purple, SNAP-25 labelled in orange.* ***(a)*** *Botox® 30U/mL* ***(b)*** *Saline control 0U/mL* ***(c)*** *Toxin 30U/mL* ***(d)*** *Buffer control 0U/mL.*

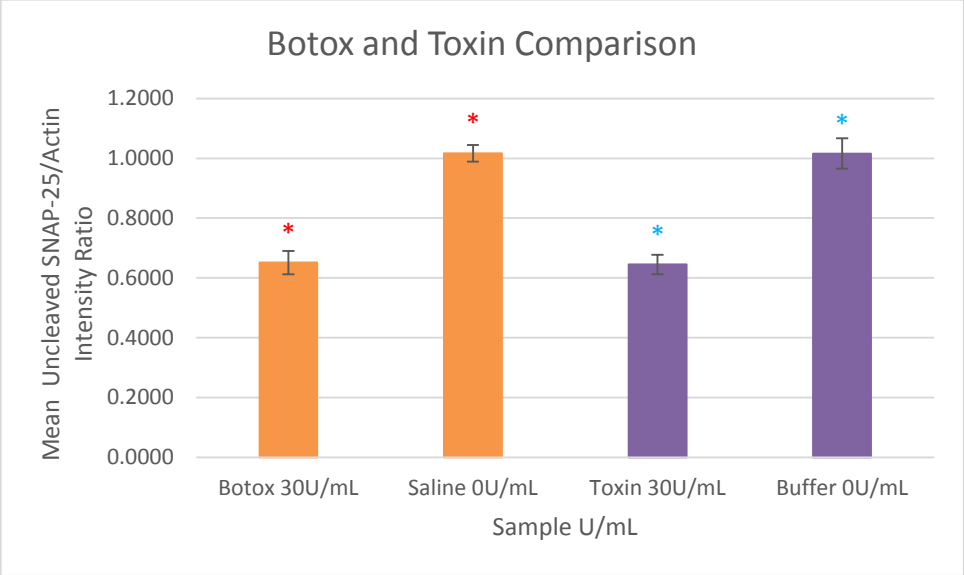


Figure 5.6: Toxin Efficiency Comparison; Botox® and Supplied Toxin. (a) (b) *Bar chart represents the uncleaved SNAP-25/Actin intensity ratio normalised to the 0U control (SNAP-25/Actin/0U control ratio). N=3, two-way ANOVA p-value= 0.00018. * indicates which data is statistically significant (saline and buffer when compared to the toxin data).*

Figure 5.5 demonstrates a comparable lack of detection of cleaved SNAP-25 to the previous experiments, suggesting that it is not an effect of the toxin. Data from three replicate experiments, shown in the graph in figure 5.6, suggests that Botox and the supplied toxin are providing comparable results and the makeup of the supplied toxin does not have any detrimental effect on the detection of SNAP-25. Statistical analysis using a two-way ANOVA provided an expected p-value of 0.00186, confirming that the toxin does influence the amount of SNAP-25 detected; suggesting cleavage. The differences in the amount apparently cleaved are minimal when comparing Botox and Toxin as shown in the pie charts in figure 5.7 below, confirming that the supplied toxin and its diluent buffer do not have any adverse effect on toxin activity or cell sensitivity. The pie charts in figure 5.7 demonstrate the percentages of cleaved and uncleaved SNAP-25 of both Botox® and Toxin, from one replicate experiment. Only the uncleaved SNAP-25 was detected, so the percentage of the cleaved SNAP-25 is assumed by the quantification of detected uncleaved SNAP-25 to the SNAP-25 detected in the control saline or toxin buffer samples.

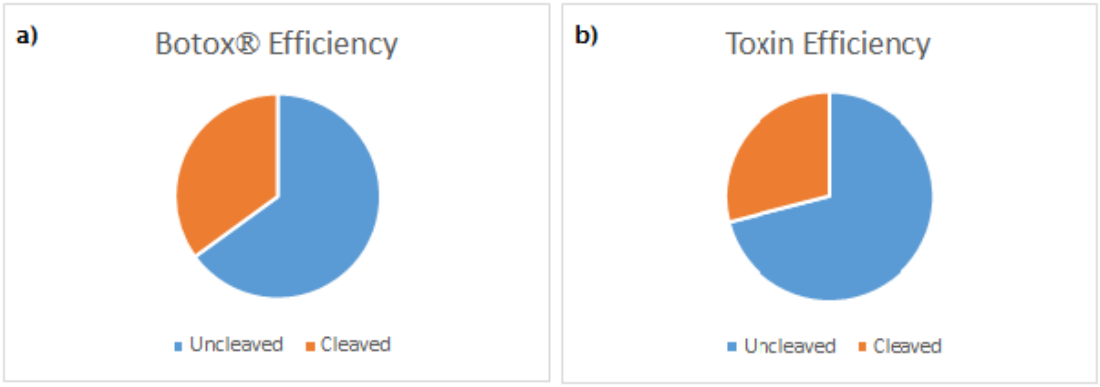


Figure 5.7: Toxin Efficiency Comparison; Botox® and Supplied Toxin. *Data calculated using the quantified uncleaved SNAP-25 detected and the SNAP-25 detected in the comparable control.* ***(a)*** *Pie chart demonstrating Botox® cleavage of SNAP-25 65% uncleaved SNAP-25* ***(b)*** *Pie chart demonstrating supplied toxin cleavage of SNAP-25 71% uncleaved SNAP-25.*

The 65% uncleaved SNAP-25 detected in the Botox® sample suggests that it is more effective in cleaving SNAP-25 (35% of SNAP-25 cleaved) compared to the supplied toxin in which 71% uncleaved SNAP-25 was detected (29% of SNAP-25 cleaved). However, analysis of three replicates has shown that the difference in detected uncleaved SNAP-25 in the two toxin samples is not statistically significant.

5.3.4 Effect of Toxin and Diluent- iCell Neurons

To demonstrate further that the toxin does not have an adverse effect on the experimental design and subsequent detection of SNAP-25 the density, viability and growth of the iCell neurons was assessed. If there is high cell death on addition of toxin this could contribute to the amount of SNAP-25 expressed in each sample. Images were captured after 48 hours of exposure to toxin and the cell density was quantified. Untreated, toxin buffer incubated and toxin exposed cell densities were compared. Figure 5.8 portrays the approach to calculating cell density; cell images from each of the assessed conditions were captured after 48-hours of exposure and the cells were manually counted using the box shown on figure 5.8a. Three images for each condition; untreated, toxin buffer and toxin exposed, were analysed, counting three squares per image and the mean for each condition is shown in figure 5.9. The standard error of the mean (SEM) is shown with the error bars, and the variance between each image analysis was minimal. The images in figure 5.8 show that there is no apparent effect on the morphology of the iCell neurons with the addition of Botulinum toxin type-A or its diluent buffer; the cells remained adherent, with the same provision of neurite processes clearly visible. The calculated p-value of 0.42 confirms that the difference in cell number across the three conditions is statistically insignificant, suggesting that the toxin or its buffer does not have a detrimental effect on the iCell neurons.

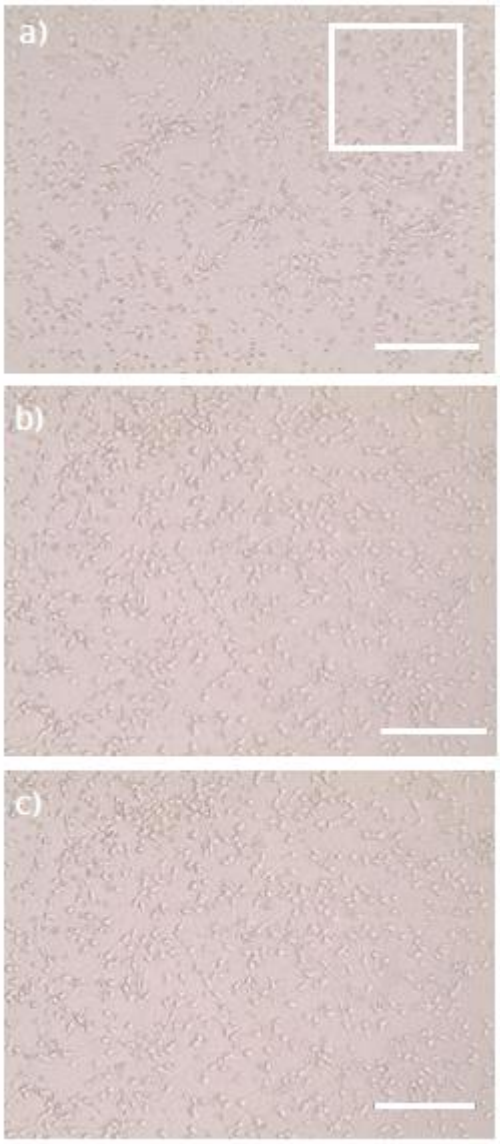


Figure 5.8: iCell Neuron Images (a) *Untreated iCell neurons* ***(b)*** *BoNT-A treated iCell neurons 30U/mL 48 hours toxin exposure* ***(c)*** *Toxin buffer control, 0U/mL 48-hour incubation. Scale bar 100µm. Images captured at x20 field of view. Captured after 48-hours of exposure. Cells manually counted using box shown on image 5.6a. Three images for each treatment (untreated, toxin and buffer conditions) were analysed, counting three squares per image and the mean calculated for each.*

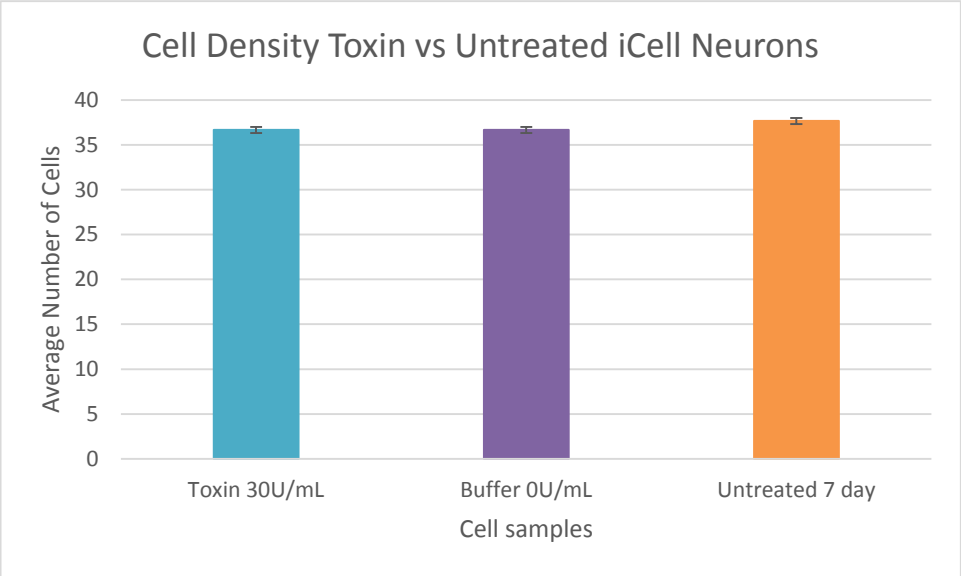


Figure 5.9: Quantification of Cell Density after Toxin Exposure. *Bar chart representing the average number of healthy cells per image in the untreated, buffer treated or toxin treated conditions. Bars represent the mean number of cells and error bars represent the standard error of the mean. N=3 independent experiments, with 3 pictures analysed per treatment. p=0.42 with one-way ANOVA test.*

To summarise, figure 5.8 images demonstrate that the cells exposed to toxin and control buffer looked as healthy and viable as the untreated cells in comparison. Only the viable cells were counted when analysed, and quantifying the number of cells did not show any statistical difference between the different treatments (figure 5.9) with a p value of 0.42. This demonstrates that the 48-hour exposure to the buffer or incubation of cells in the toxin does not seem to affect cell number.

5.3.5 ReproCell Toxin Experiments

ReproCell ACH and DA toxin treated cell samples (30U/mL and 0U/mL control) were collected, as explained in the methodology section, and analysed for toxin sensitivity with Western Blot. Uncleaved SNAP-25 was detected in both DA and ACH cell lines, and results from three replicates were quantified.

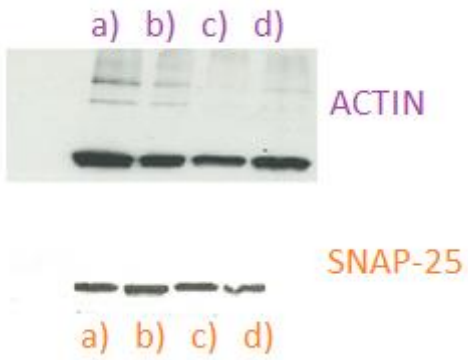


Figure 5.10: ReproCell ACH and DA Western Blots. *The blot shows bands detected in ReproCell ACH and DA toxin sensitivity experiments. Actin labelled in purple, SNAP-25 labelled in orange.* ***(a)*** *DA 30U/mL* ***(b)*** *DA 0U/mL* ***(c)*** *ACH 30U/mL* ***(d)*** *ACH 0U/mL. SNAP-25 detected at 25kDa and Beta-Actin at 42kDa.*

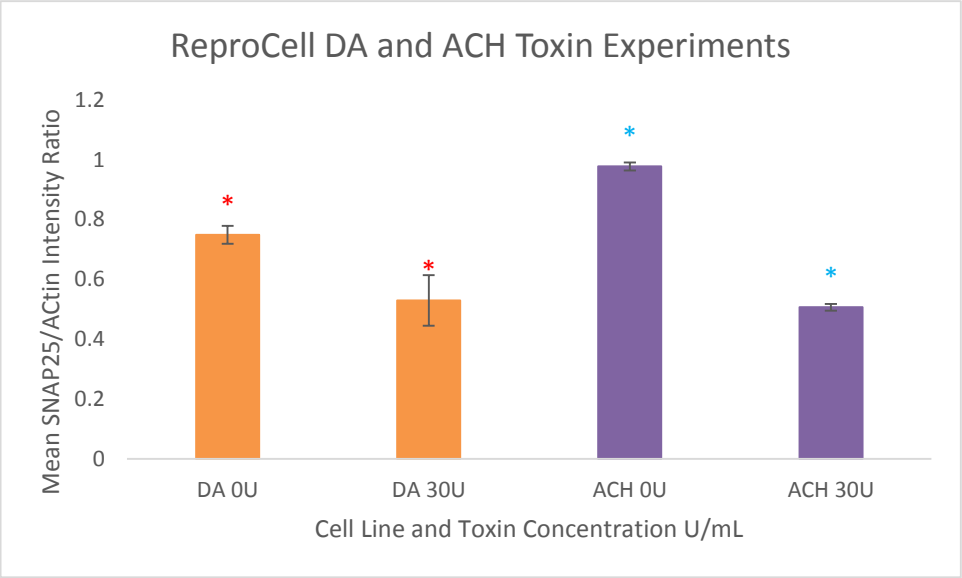


Figure 5.11: Bar chart representing the comparison of DA and ACH ReproCell samples as untreated (0U/mL) and toxin treated (30U/mL). *The bars represent the mean, of three replicates, (one vial of cells) uncleaved SNAP-25/Actin intensity ratio, and the error bars show the SEM of these figures. Two-way ANOVA p=0.00195. * indicates which data is statistically significant (saline and buffer when compared to the toxin data). No statistical difference between toxin performance for each cell line.*

As demonstrated by the blot shown in figure 5.10, only uncleaved SNAP-25 was detected in the analysis of the ReproCell cell lines. The Western Blot analysis provided the data for the graph shown in Figure 5.11, demonstrating an apparent cleavage in SNAP-25 following incubation in Botulinum toxin type-A. Statistical analysis with a two-way ANOVA suggested a p-value of 0.001945, suggesting significance between the treatments (0U and 30U), supporting the indication of SNAP-25 cleavage. The ACH cell line analysis indicated that 49% of SNAP-25 was cleaved; with a detection of 51% of uncleaved protein, when normalised to the 0U control. The DA cell line demonstrated a highly reduced cleavage, based on the detection of 71% of the uncleaved SNAP-25 protein, suggesting a small 29% cleavage. Based on these results, it can be hypothesised that the ReproCell ACH cell line has the potential to be more sensitive than the ReproCell DA cell line. With reference to Chapter 4, a repeat of the experiments, with a new batch of the commercially available cells indicated that SNAP-25

expression is varying in the DA samples. Figure 4.23 shown in Chapter 4 shows a blot analysis of the Reprocell samples, in which SNAP-25 is abundant in the ACH cells, but barely detectable in the DA. It could be hypothesised that the toxin cleaved all SNAP-25, but the low detection of SNAP-25 in the 0U control suggests low abundance of expression.

5.3.6 Western Blot Optimisation for Detection of Cleaved SNAP-25

The iCell experiments provided comparable results to the published paper (Whitemarsh, 2012) in terms of cell sensitivity and expression of SNAP-25 but detection of the cleaved SNAP-25 band, which sits at 24kD just below SNAP-25, was proving to be a challenge. Even with increased antibody concentration, the band could still not be revealed. The preliminary results suggested the hypothesis that iCell neurons are sensitive to Botulinum Toxin, due to decrease of the uncleaved SNAP-25 band intensity, but detection of the cleaved band would provide a more robust result, so optimising the Western Blot technique was fundamental. The gels were changed from 12% TGX gels to 4-20% gradient gels to provide a greater separation of bands at the base of the gel. With these, we were able to successfully detect the cleaved band, separately from the uncleaved SNAP-25 at 25kDa. Cooling the running and transfer buffers and increasing the time for protein migration through the gel improved separation, altering the method to incorportate migrating the proteins until the 15kD marker was at the base of the gel. The detection of the cleaved band is shown in the blot in figure 5.12 below, detected using the primary mouse monoclonal antibody from Synaptic Systems at a concentration of 1:1000.

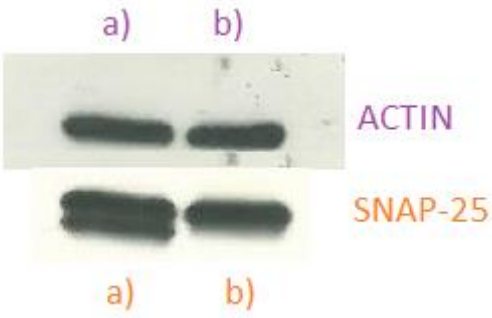


Figure 5.12: Detection of Uncleaved and Cleaved SNAP-25 in iCell neurons. *Blot revealing the cleaved SNAP-25 band at 24kDa below the uncleaved 25kDa band. SNAP-25 labelled in orange, Actin in purple.* ***(a)*** *SNAP-25 cleaved and uncleaved 30U/mL* ***(b)*** *SNAP-25 whole 0U/mL.*

This figure clearly shows a separate protein band just underneath the 25kDa band, sitting at 24kDa representing cleaved SNAP-25 as a result of toxin activity. Successful separation of the two forms of SNAP-25 allows for proper quantification and assessment of toxin activity. The experimental design of 48-hour toxin incubation at 30U/mL was carried out on the iCell neurons with the adaptations to the Western Blot methodology, and the results are shown below.

5.3.7 Repeat of iCell Experiments with Detection of Cleaved SNAP-25

Following successful detection of the SNAP-25 cleaved protein band the 48-hour toxin incubation at 30U/mL was repeated. Given the high cost of the cell line, it was not deemed economically beneficial to repeat all of the previous experiments; and the results below show similar predictions made from the experiments in which only the uncleaved form of SNAP-25 was detected.

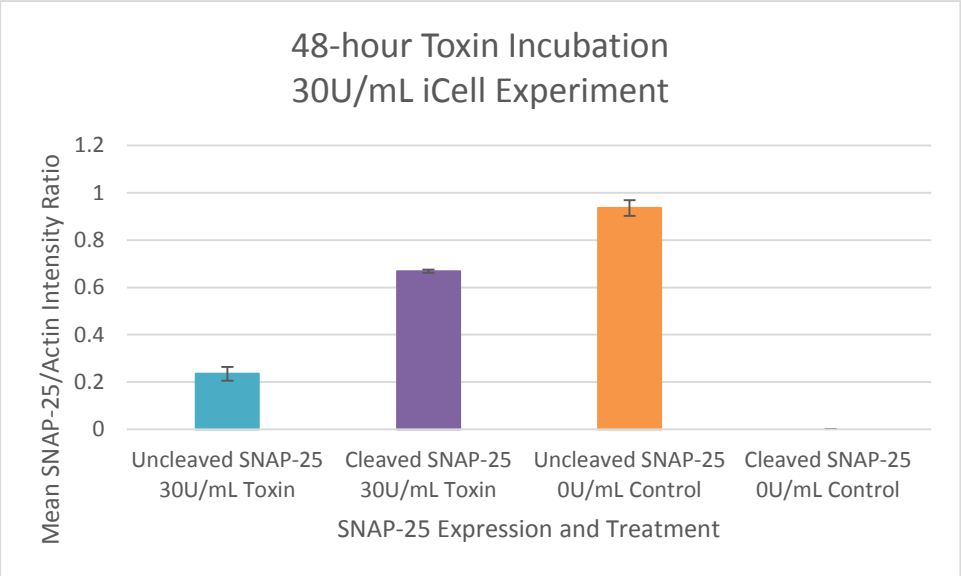


Figure 5.13: Bar chart representing SNAP-25 cleavage in iCell neurons. *Cells incubated in 30U/mL toxin (uncleaved and cleaved SNAP-25 bars) or 0U/mL control (buffer- whole SNAP-25 quantified). The bars represent the mean of three replicates, (one vial of cells) SNAP-25/Actin intensity ratio, and the error bars show the SEM of these figures. N=3. T-test Cleaved SNAP-25 p=0.0012 T-test Uncleaved SNAP-25 p= 0.0076.*

The t-tests analysis indicated statistically significant p-values for both the comparison of the uncleaved toxin treated to the uncleaved control and the cleaved SNAP-25 in the toxin treated sample to the cleaved in the control. We would expect there to be statistical significance when comparing these treatments which allows the acceptance of the hypothesis that the introduction of toxin to the cells induces cleavage of SNAP-25.

The quantification of the Western Blot data collated in the graph in figure 5.13 above indicates that the cleaved portion of SNAP-25 is over 50%; suggesting an improvement in the results presented previously with only the uncleaved portion of SNAP-25 available for analysis. If the 0U/mL control is accepted as an indication of total SNAP-25 in the cell samples analysed, then the incubation of cells in 30U/mL of toxin for 48-hours yielded a 68-75% cleavage of SNAP-25. Percentages of SNAP-25 cleaved calculated from three separate cell samples are shown in figure 5.16 below, each treated with 30U/mL of toxin for 48-hours.

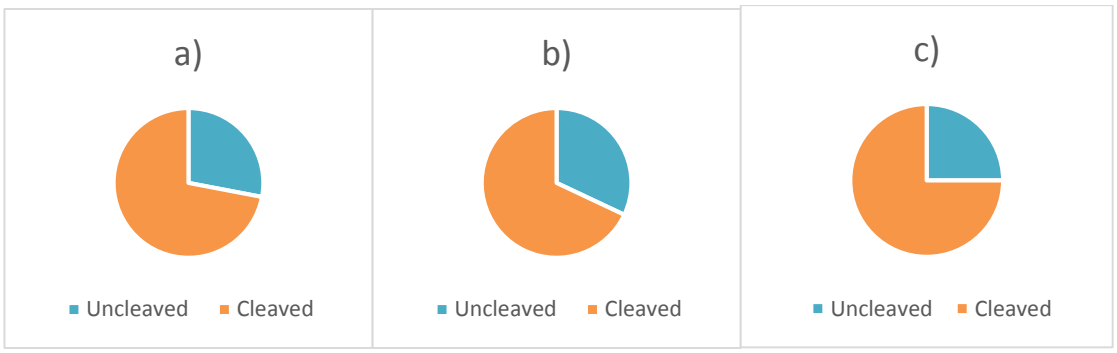


Figure 5.14: Pie Chart Representation of the Percentage of SNAP-25 cleaved following toxin exposure. *Three replicate cell sample percentages are shown (a) 72% SNAP-25 cleavage (b) 68% SNAP-25 cleavage (c) 75% SNAP-25 cleavage.*

The consistency of the cleavage of SNAP-25 is demonstrated with each replicate and confirms that the 48-hour toxin incubation is reliable and ensures the accumulation of cleaved SNAP-25 at a detectable limit using Western Blot analysis.

5.3.8 Assessing Toxin Activity

Following optimisation of the Western Blot methodology to ensure detection of both cleaved and uncleaved SNAP-25, the analytical comparison of Botox® and the supplied toxin was reanalysed. Detection of both cleaved and uncleaved SNAP-25 in both the Botox® and toxin treated samples were quantified and shown in the following graph, figure 5.15. The control samples of saline and toxin buffer were analysed alongside the toxin treated samples.

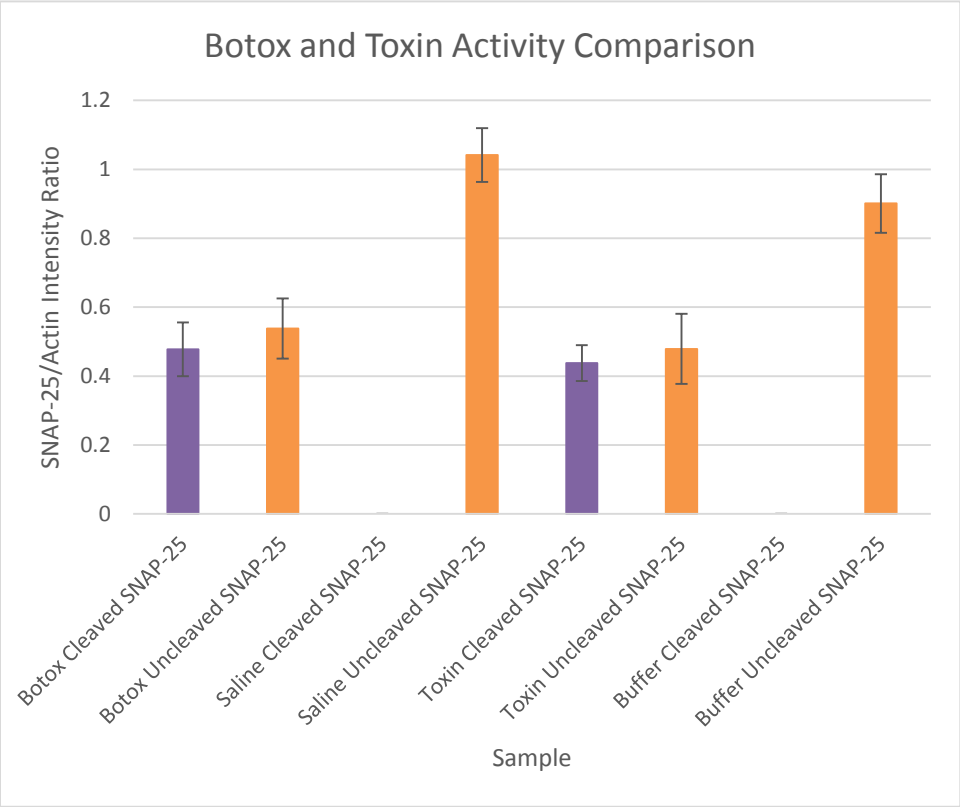


Figure 5.15: Toxin Efficiency Comparison. *Bar chart representing the quantified SNAP-25/Actin intensity ratio in Botox® treated and toxin treated cells; each with a corresponding control in which the buffer diluent was added. 2-way ANOVA for Cleaved SNAP-25 p= 0.0397 2-way ANOVA for Uncleaved SNAP-25 p= 0.0205.*

Statistical analysis using the 2-way ANOVA method to compare the cleaved SNAP-25 results for both Botox and toxin indicated a p-value of 0.0397, suggesting significance. This allowed the acceptance of the hypothesis that both Botox and toxin influence the cleavage of SNAP-25 when compared to the control samples for each toxin. The p-value of 0.0205 for the uncleaved SNAP-25 suggests similar predictions that there is a significant difference in the amount of SNAP-25 uncleaved in the toxin treated samples when compared to the 0U.mL controls.

The improved Western Blot methodology allowed for quantification of both cleaved and uncleaved SNAP-25. A replicate of the Botox® and toxin comparative data is shown in figure 5.15 bar graph, which shows that incubation with both 30U/mL Botox® and 30U/mL toxin drug product provide comparable cleavage of SNAP-25. This confirms the efficiency of the supplied toxin drug product

matches a similar commercially available product. Previous experiments shown in figure 5.7 indicated that Botox® presented a 35% cleavage of SNAP-25 (65% uncleaved SNAP-25 detected) and the toxin analysis suggested a reduced 29% cleavage (71% uncleaved SNAP-25 detected). The repeat of the experiments with the detection of both cleaved and uncleaved SNAP-25 separately demonstrates a much higher percentage of SNAP-25 cleavage. The Botox® shows a mean of 54% SNAP-25 cleavage, with the supplied toxin demonstrating a slightly improved mean of 57% cleavage of SNAP-25. These percentages are graphically demonstrated in the pie charts in figure 5.15 below.

Figure 5.16: Toxin efficiency comparison; Botox® and supplied toxin. *Data calculated using the quantified uncleaved and cleaved SNAP-25 detected and the SNAP-25 detected in the comparable control.* ***(a)*** *Pie chart demonstrating Botox® cleavage of SNAP-25 54% cleaved SNAP-25* ***(b)*** *Pie chart demonstrating supplied toxin cleavage of SNAP-25 57% cleaved SNAP-25.*

5.4 Discussion – Toxin Sensitivity of Cell Lines

The design of the experiments completed for analysis of toxin sensitivity were aimed at discovering the optimum conditions for using the cell line for a cell-based assay for the potency testing of Botulinum toxin type-A. At this stage in the project, the iCell neurons were posed at the most positive candidate, with consistent expression of SNAP-25 demonstrated in chapter 4. The ReproCell ACH and DA samples were also analysed but sample availability was limited, given the issues with cell density, discussed previously. The experimental analysis of the iCell neurons allowed for the selection of the optimum toxin testing conditions to utilise the available sample from the ACH and DA cell lines.

Toxin Dose Response and Effect of Exposure Time: Based on the comparative data obtained when comparing the exposure times of 6-hours and 48-hours, the latter was selected as the optimum toxin incubation time for the accumulation of detectable SNAP-25. The 6-hour exposure time provided a maximum of 43% SNAP-25 cleavage with the 500U/mL toxin treatment, whereas preliminary experiments in which only the uncleaved portion of SNAP-25 was detected, 48-hour exposure to toxin allowed for a 54% cleavage of SNAP-25. The 54% cleavage was achieved using 30U/mL of toxin and this was set as the optimal concentration for analysis of the ReproCell cell lines. This is the concentration of toxin that induces a response halfway between the baseline and maximum of the control (EC50). Further results from the 6-hour experiment concluded that the 100U/mL toxin exposure yielded a cleavage of 36% and the 1000U/mL a reduced 23% cleavage. This suggests that perhaps the cells need longer than 6-hours to cleave larger percentages of SNAP-25. If you compare these figures to the 41% SNAP-25 cleavage with 10U/mL of toxin for 48-hours, and the optimal 30U/mL with a cleavage of 54% of SNAP-25 it is clear that the amount of SNAP-25 cleaved increases with exposure time. The slight anomaly in this data was shown with the 0.1U/mL for 48-hours condition, with a large standard error of the mean suggesting the replicates were not consistent. This yielded a mean of 20% cleavage over 48-hours, a significant reduction in comparison to the other doses analysed at 48-hour exposure.

However, these results were calculated based on the detection of uncleaved SNAP-25 only, and development of the Western Blot later allowed for the 30U/mL for 48-hours experiment to be repeated. Given the expense of the iCell neurons,

it was decided to only repeat the suggested most efficient time point and toxin dose based on the previous data; 30U/mL for 48-hours. The results provided a clear indication that this toxin concentration was much higher than 50% of the baseline to the maximum (EC50) and replicate experiments yielded 68-75% SNAP-25 cleavage, with detection of both the cleaved and the uncleaved forms of the protein.

Toxin Activity and Media Comparison: The results shown in figure 5.4 demonstrate that the toxin cleaves approximately the same amount of SNAP-25 in the neuronal media at the 1 or 10-minute time points, but the comparable stimulation media data suggests a clear increase in SNAP-25 over the time points. This experiment shows that the cells can internalise and translocate toxin as soon as 1 minute after exposure. Although the results indicate that there is an increase in toxin activity in the stimulation media when comparing 1 to 10 minutes exposure, statistical analysis confirms that the difference in the results is not significant. Based on these observations, and regarding the use of expensive iCell neurons in particular, to reduce the number of reagents, and thus potential variables, to the media make-up is the better option; especially as there was no significant improvement in toxin activity with the use of stimulation media. These results, when combined with the assessment of toxin exposure time discussed previously, suggest that although the cells are showing uptake of toxin after 1-minute exposure, the accumulation of cleaved SNAP-25 is optimum after 48-hours. When comparing the SNAP-25 detected at 6-hours compared to that at 48-hours, the latter provided improved results, suggesting a longer exposure time allows for more toxin cleavage.

Effect of Toxin on Cell Viability: As the detection of the SNAP-25 cleaved band was not visible with the preliminary Western Blot analysis, experiments to eliminate the possibility of the toxin preparation buffer and formulation interference with the detection capabilities were carried out. Several experiments were carried out to assess possible reasons as to why only the uncleaved portion of SNAP-25 could be detected and it was proposed that reduced cell viability could be influencing the expression of SNAP-25, as a result of toxin or toxin diluent buffer exposure. iCell neurons were observed under the microscope on addition of toxin and toxin diluent buffer to measure any adverse effect on the cell population. Images were captured and healthy cells were counted to provide comparable data for cells incubated in toxin or toxin buffer to those in

maintenance media, or untreated cells. A p-value of 0.42 shows that there is no significant statistical difference in the number of cells in each of the three conditions, suggesting that the toxin and buffer do not have a detrimental effect on cells when they are exposed to the reagents for 48-hours. Cell death and reduced viability could at this stage be eliminated as the cause of undetectable cleaved SNAP-25. The secondary analysis of potential detrimental effect on cells caused by the provided toxin was to compare the efficiency and expression of SNAP-25 with a commercially available product that has been previously shown in research to act in the same way as the provided toxin when added to cell culture.

Toxin Efficiency Comparison: A comparison of commercial Botox® and the supplied toxin, and comparable buffers would indicate whether the buffer was degrading the cleaved SNAP-25 protein and was a second attempt to reduce the possible reasons for the inability to detect cleaved SNAP-25. Comparing the toxin efficiency to that of a commercial product provided the opportunity to compare toxin efficiency and ensure that protein degradation was not a factor. The preliminary analysis of both Botox® treated and toxin treated samples, together with the controls of saline and diluent buffer, again only provided detection of the uncleaved portion of SNAP-25. Based on these results it was concluded that Botox® presented a 35% cleavage of SNAP-25 and the supplied toxin cleaved less at 29% cleavage of SNAP-25. This further clarified that the toxins did not have an adverse effect on cell density by inducing cell death which in turn could lead to reduced SNAP-25 expression, and it was not a possibility that the toxin had degraded the cleaved portion to an undetectable limit. Botox® has been shown in published literature to allow for the detection of and distinction between both cleaved and uncleaved SNAP-25 in the same treated sample. Further optimisation of the Western Blot methodology allowed for the eventual detection of both forms of SNAP-25 following toxin activity and the comparison of Botox® and the supplied toxin was repeated. The results indicated that detection of both cleaved and uncleaved SNAP-25 was possible for both toxin treated samples, using 30U/mL and exposing the cells for 48-hours. The results indicated a vast improvement in SNAP-25 cleavage; the supplied toxin yielding a 57% cleavage of SNAP-25 at 30U/mL and Botox® a 54% cleavage. This was possibly due to the quantification of merged uncleaved and cleaved SNAP-25 bands, rather than individual quantification of each form. This proved

that our supplied toxin is as efficient as other commercially available drug products and the results confirm the efficiency of the product.

The ability to be able to detect both cleaved and uncleaved forms of SNAP-25 enables the conclusion that the hiPSC-derived neurons (iCells) have optimal SNAP-25 expression after 7 days of culture (chapter 4) and that 48 hours of toxin exposure provides the optimal accumulation of cleaved SNAP-25. 30U/mL toxin exposure provided the optimum results and was selected as the most efficient for use on screening the ReproCell ACH and DA cell lines. 48-hour exposure has no detrimental effect on cell viability and ability to internalise toxin, and repetition of the results of the 30U/mL concentration and 48-hour exposure allowed detection of both cleaved and uncleaved SNAP-25 following Western Blot optimisation.

ReproCell ACH and DA Cell Line Analysis: The analysis of the two ReproCell cell lines was carried out following confirmation of apparent optimum toxin concentration and exposure time, demonstrated in the analysis of the iCell neurons samples. The sample availability was limited for both the ACH and DA cell lines, with issues surrounding cell density as discussed previously, and the best option for analysis was selected; 48-hour toxin incubation at a concentration of 30U/mL. The analysis was carried out before the optimisation of the Western Blot methodology so the results are only based on the detection of uncleaved SNAP-25. The ACH cells rendered a 49% cleavage of SNAP-25, comparative to results shown when analysing the iCell neurons initially, and the DA cell line yielded a SNAP-25 cleavage of 29%. Following detection method adjustments, there was not enough sample remaining to carry out sufficient analysis, and as the iCell neurons were providing accurate and consistent results, further analysis of the ReproCell cell lines was ceased. Considerations were made regarding the variability in cell number, the variability in the expression of SNAP-25 as discussed in chapter 4 the ReproCell ACH and DA cell lines were not considered for further analysis for use in the cell-based assay.

The optimisation of the Western Blot methodology is discussed in detail in chapter 7. Migration time was increased and this allowed for the successful detection of both cleaved and uncleaved SNAP-25 in the same samples.

	Principle/Standard	Cell Line Experiments	Action
1	Toxin Exposure- Incubation Time Comparison	6-hour incubation x	Select optimal incubation time for further experiments **48-hours**
		48-hour incubation ✓	
2	Toxin Uptake/Media Comparison	Stimulation media ✓	Select optimal medium for future experiments Difference statistically insignificant
		Neuronal media ✓	
3	Distinction between Cleaved and Uncleaved SNAP-25	Both isoforms of SNAP-25 are detectable ✓ x x	Proceed with cell line- suitable selection iCell Neurons
		Only uncleaved SNAP-25 is detectable x ✓ ✓	Optimise method or eliminate cell line as a suitable candidate ReproCell ACH/DA
4	Repeatability of toxin treatment conditions	Cell line provides consistent and repeatable results. ✓ x x	Consider cell line for criteria 4 analysis (expression of receptors) iCell Neurons
		Cell line is variable, results are inconclusive x ✓ ✓	Eliminate cell line as a suitable candidate ReproCell ACH/DA

Table 5.9: Principles and Requirements for Consideration for Criteria 3; Toxin Sensitivity. *The tabled criteria are considerations when analysing each of the selected cell lines toxin sensitivity. Observations for each remaining cell line are shown in colour- ReproCell ACH, ReproCell DA, iCell neurons*

The principles and standards defined in the introduction to this chapter were assigned as essential requirements for the selection of a suitable cell line for the cell-based assay. When applying these principles to the experiments in chapter 5 it can be concluded that 48-hour toxin exposure provides the optimum results for assessing toxin activity and that the statistically insignificant data concluded from the comparison of media experiments suggested no improvement of toxin activity and subsequent cleavage with the use of stimulation media. Considering the cost of the iCell neurons, it was decided to keep the maintenance protocol as similar to that recommended by the supplier as possible, to reduce the risk of any detrimental effect on the cell product. Therefore, the use of supplied neuronal media, with no additional reagents or altered concentrations, was selected for use in all further experiments and in the finalised cell-based assay. The distinction between cleaved and uncleaved SNAP-25 was an essential element that allows for the quantification of toxin activity, and this was achieved with the optimisation of the Western Blot method, and these two forms of SNAP-25 were distinctly shown in the iCell neurons. This could not be achieved with the ReproCell ACH and DA cell lines due to lack cells, but this is not the only suggestion that the cells are not suitable for the cell-based assay. Moreover, the repeatability of experiments and toxin treatment conditions principle could also not be applied to the ReproCell cell lines. The variability of cell number, without the possibility of producing a greater cell population, and the inconsistent detection of SNAP-25 demonstrated by the DA cell line allowed for the elimination of both the ACH and DA cells for use in the testing of Botulinum toxin. This leaves the remaining, and shown to be most sensitive cell line, the iCell neurons as the most suitable cell line for potency testing of botulinum drug product.

5.5 Conclusion – Toxin Sensitivity of Cell Lines

The data shown in this chapter indicates that the hiPSC-derived neurons (iCells) are the most sensitive of the selected cell lines to botulinum toxin type A. The toxin does not have an adverse effect on the cells at concentrations as high as 1000U/mL, and SNAP-25, both cleaved and uncleaved, is present at a detectable level with antibody analysis via Western Blot. The cleavage of SNAP-25 concludes that the iCells express the SNARE receptors ideal for intoxication with botulinum toxin and a comparison by Whitemarsh, et al, 2012 in their paper of the iCell neurons to primary rat spinal cord cells, the iCells demonstrate equal or greater sensitivity (Whitemarsh, Strathman et al. 2012). The results discussed in chapters 3, 4 and 5 have led to the conclusion that the iCell neurons are sensitive to toxin, can reliably express SNAP-25, are easy to maintain and culture and the results from each experimental condition have been repeatable and consistent. They are an excellent candidate for use in the final complete assay for the *in vitro* potency testing of Botulinum toxin type-A and provide a potential platform for the design of a highly-specific and reliable assay method when combined with a sensitive detection method. Table 5.10 below summarises all of the previous analyses of the cell lines and shows clear indication that the iCells are by far the most efficient, sensitive and suitable for the project requirements.

Cell Line		hNSCs	BE(2)-C	SH-SY5Y	IMR-32	ACH	DA	iCells
Criteria 1: Ease of Culture and Maintenance (Chapter 3)								
Proliferation		✓✓	✓✓✓	✓✓✓	✓	✓	✓	✓
Culture Maintenance		✓✓	✓✓	✓✓	✓✓✓	✓✓	✓✓	✓✓✓
Repeatability of Culture Conditions		✓	✓✓✓	✓✓✓	✓	✓	✓	✓✓✓
Criteria 2: Expression of SNAP-25 (Chapter 4)								
Expression of SNAP-25	MS	---	✓	---	✓	---	---	x
	WB	x	x	x	x	✓✓	✓	✓✓✓
Further analysis?		x	x	x	x	✓	✓	✓
Criteria 3: Toxin Sensitivity (Chapter 5)								
Distinction of Cleaved and Uncleaved		n/a	n/a	n/a	n/a	x	x	✓
Repeatability		n/a	n/a	n/a	n/a	x	x	✓
Further Analysis		No	No	No	No	No	No	Yes

Table 5.10: Summary of Previous Conclusions for Cell Line Screening. *These criteria led to the selection of the most sensitive cell line for use in the cell-based assay; iCell Neurons. For criteria one, one tick to three ticks progressively indicate the level of ease; one is difficult to maintain or slow-growing and three ticks is very easy to maintain and high proliferation rate. Criteria two and three are ticked or crossed according to observations made on success of SNAP-25 expression or toxin sensitivity.*

Cell Line	ACH	DA	iCells
Toxin Sensitivity	?	?	✓✓✓
Further Analysis?	×	×	✓

Table 5.11: Selection of the iCell Neurons *(Cellular Dynamics)*

Chapter 6: Receptor Expression

6.1 Introduction

The results discussed in Chapters 3-5 have led to the conclusive selection of the iCell neurons as the cell line most suitable for use in a cell-based assay for the potency testing of Botulinum toxin type-A. Western Blot analysis has clearly shown that the toxin actively cleaves SNAP-25 and this proven toxin activity suggests that the iCell neurons express the receptors necessary for the successful binding and toxin entry into the cell. To show expression of the receptors specifically important for the toxin sensitivity would further support the section of the iCell neurons for our projects use. It has been shown that BoNT-A interacts with the glycoprotein SV2, which is localised to synaptic vesicles (Mahrhold, Rummel et al. 2006) (Sudhof 2004). As described in Chapter 1, there are three isoforms of SV2; A. B and C, but it is suggested that BoNT-A interacts predominently with SV2C (Dong, Yeh et al. 2006, Mahrhold, Rummel et al. 2006).

Toxins have been shown to bind to polysialogangliosides as well as protein receptors and it has been demonstrated that heavy chain (HC) fragments of botulinum toxin type A bind primarily to Gt1b (Rummel, Mahrhold et al. 2004). The addition of gangliosides has been shown to increase the level of coimmunoprecipitation of BoNT-A with SV2 receptor proteins (Dong, Yeh et al. 2006).

Pre-treatment of cell cultures with gangliosides increases the sensitivity to BoNT-A (Bullens, O'Hanlon et al. 2002) and this addition to the culturing methodology has been utilised by a cell-based assay that has been submitted by another company in previous months. This element has been included in the patent for this submitted assay, and therefore may prove problematic if utilised by the project cell-based assay. The improvement of sensitivity to toxin with the addition of Gt1b was proposed for analysis and is discussed in this chapter; with the suggestion to the sponsoring client that if it improves sensitivty dramatically the issues surrounding IP could potentially be addressed.

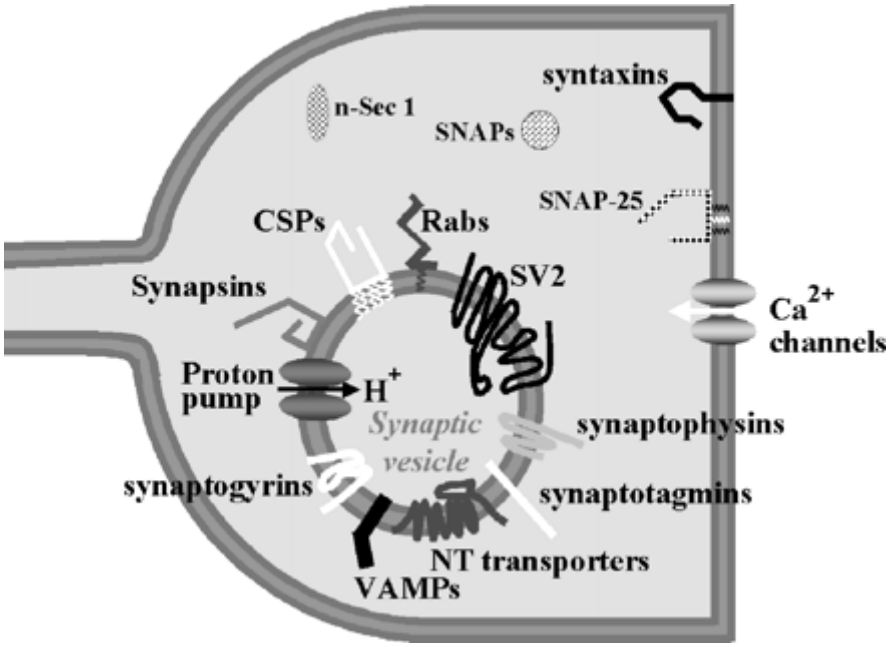


Figure 6.1: Schematic of a Presynaptic Terminal showing Presynaptic Proteins. *Image taken from (Staple, Morgenthaler et al. 2000) Presynaptic Heterogeneity: Vive la Difference. SV2 is shown to be on the membrane of a synaptic vesicle.*

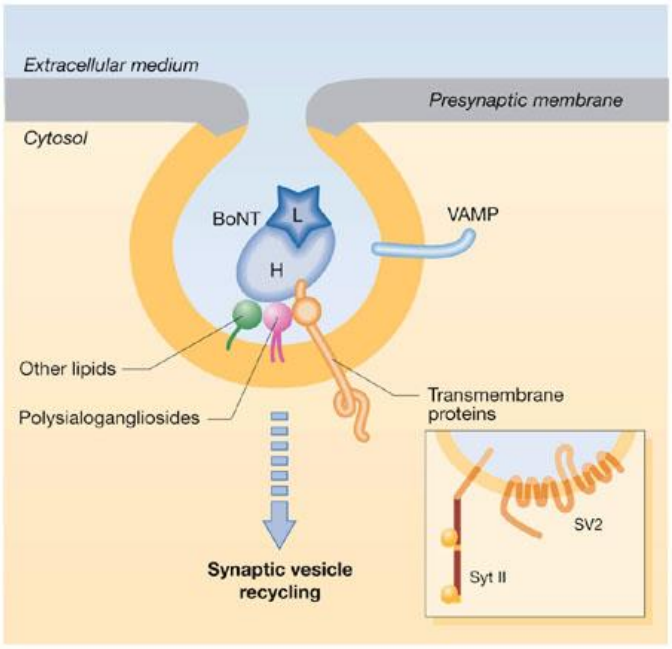


Figure 6.2: Toxin Binding in the Synapse. *This diagram illustrates the role of gangliosides and synaptic vesicle protein in mediating botulinum toxin binding and entry into neurons. (Verderio, Rossetto et al. 2006)*

It is proposed that receptors for BoNTs are composed of both gangliosides, with low affinity binding and protein receptors that form high-affinity complexes with toxins (Dong, Yeh et al. 2006) (Rummel, Mahrhold et al. 2004). A study aimed to identify the protein receptors for BoNT-A by focussing on the toxins route of entry demonstrated results suggesting that synaptic vesicle exocytosis exposes the receptors required for BoNT-A binding (Dong, Tepp et al. 2007).

6.1.1 SV2A

SV2A is the most highly expressed isoform of SV2, located, in abundance, in the cerebral cortex, midbrain and cerebellum (Tokudome, Okumura et al. 2016). It is suggested that it modulates calcium dependant neurotransmitter release, thus regulating the expression of the calcium dependant protein synaptotagmin (Syt) (Madeo, Kovacs et al. 2014).

6.1.2 SV2B

It is suggested by (Morgans, Kensel-Hammes et al. 2009) that of the three isoforms of SV2, SV2B is the most conflicting in contrast to SV2A and SV2C. The research into the role of the B isoform in the binding of Botulinum toxin type-A is limited in comparison and interaction is primarily documented with the A and C isoforms. SV2B is more broadly expressed in the neonatal brain rather than the adult suggesting its role in synapse development (Morgans, Kensel-Hammes et al. 2009). A study by (Bajjalieh, Frantz et al. 1994) confirmed that where SV2A is expressed universally throughout the brain at varying levels, SV2B has a more limited distribution, changing throughout development of the brain and is found in cells that have not yet established synaptic contact.

6.1.3 SV2C

SV2 is localised to synaptic and endocrine secretory vesicles (Sudhof 2004) with SV2A expressed in virtually all synapses. SV2B is more restricted in distribution, though also found in synapses throughput the brain whereas SV2C is documented to be limited to ‘older’ brain regions (Janz and Sudhof 1999). SV2C is only found in a few areas of the brain such as the midbrain, brainstem and pallidum and undetectable in the cerebral cortex and hippocampus. BoNT-A

binds directly to a luminal loop of SV2A, B and C with SV2C showing the most robust BoNT-A binding (Dong, Yeh et al. 2006).

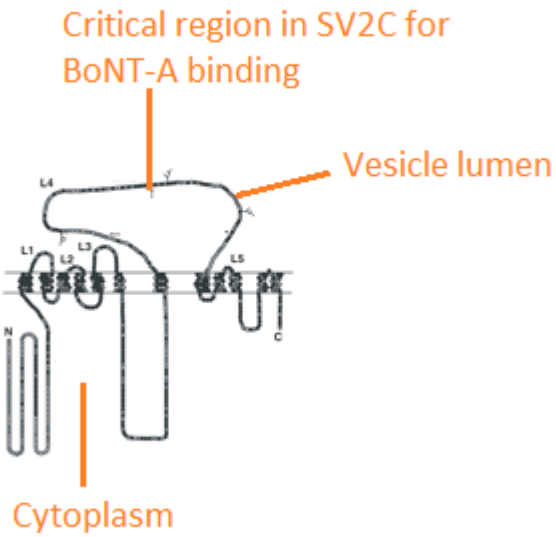


Figure 6.3: Model of SV2 Topology. *Adapted from figure shown in research paper 'SV2 is the Protein Receptor for Botulinum Neurotoxin Type-A' by (Dong, Yeh et al. 2006). Figure highlights the specific binding site for BoNT-A on the SV2C receptor.*

The three isoforms have a similar structure, which cannot be clearly determined in figure 6.3 above. A paper by (Weisemann, Stern et al. 2016) details a more specific diagrammatical figure that clearly shows the differences in residues between the three isoforms of SV2, as shown in figure 6.4 below.

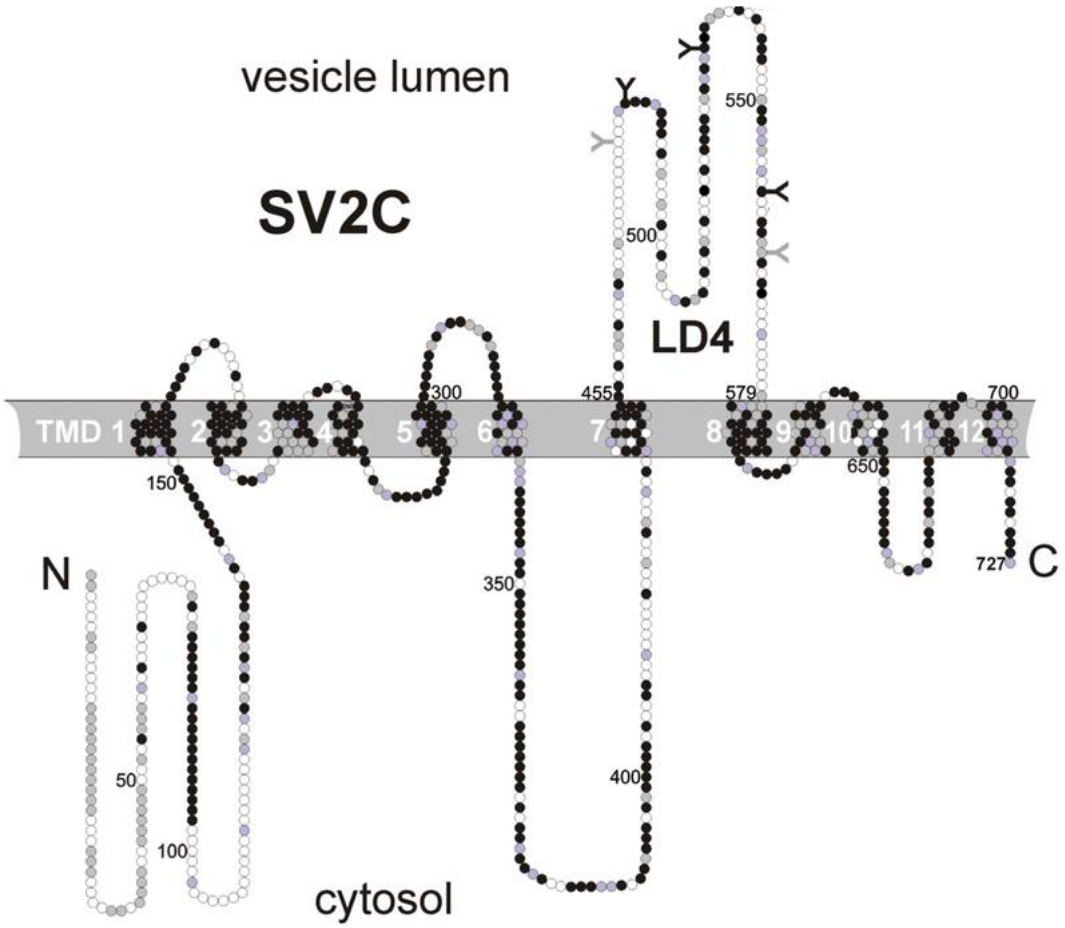


Figure 6.4: Membrane Topology of Rat Synaptic Vesicle Protein 2 Isoform C (SV2C). *Taken from (Weisemann, Stern et al. 2016). Residues identical in all isoforms are shown in black, residues specific to only SV2A and SV2B are shown in grey. The glycosylation sites of LD4 are indicated with 'Y' and the three strictly conserved to SV2C in black.*

As the role of SV2 is suggested to be essential in the binding of BoNT-A this chapter aimed to analyse the expression of both SV2 in iCell neurons; the cells have been shown to be sensitive to toxin but analysis of the expression of receptors will confirm the selection of the cell line. As it is suggested that SV2A is the most abundant and that SV2C is the most involved in the binding of BoNT-A, these two isoforms were selected for analysis. SV2B was set aside based on previous research conducted by Whitemarsh (*et al*, 2012) as shown in figure 6.5 and literature indications that this isoform is not as abundant in neurons.

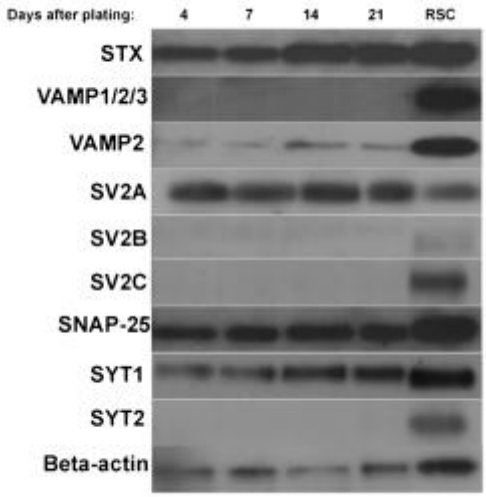


Figure 6.5: Western Blot Analysis of SNARE Proteins and SV2 Receptors. *Blot taken from Whitemarsh (et al, 2012) to show justification for analysis of only SV2A and SV2C in this projects experiments.*

The expression of SV2 was analysed using Western Blot. Peptides for the proteins were purchased from Synaptic Systems (see table 6.1 in methodology) to use as standards for the analysis of expression in the cell samples. This detection method was successful in the detection and distinction between cleaved and uncleaved SNAP-25 and therefore the Western Blot was used to analyse the SV2 proteins, using the same methodology as described in previous Chapters.

For optimisation of antibody concentrations for SV2 analysis and preliminary experiments using Gt1b as an addition to culture methods, the neuroblastoma cell lines BE(2)-C and SH-SY5Y were used before analysing iCell neurons to minimise costs and reduce the number of vials of iCells used in the optimisation of a new methodology.

6.2 Methods

6.2.1 SV2 Receptor Expression Analysis

6.2.1.1 Optimisation of SV2 Antibodies Using Neuroblastoma Cell Lines

Neuroblastoma cell lines BE(2)-C and SH-SY5Y samples were collected to analyse for SV2 receptor expression. Cells were differentiated in retinoic acid for 9 days, as described in Chapter 4, section 4.2.1 and then collected in RIPA buffer with protease inhibitors. Toxin treated samples (30U/mL with a 0U/mL control) and untreated, undifferentiated samples were also collected for analysis. These methods have been previously described in Chapters 4 and 5. Primary antibodies from Synaptic Systems were trialled at three different concentrations; 1:100, 1:1000 and 1:10000 (see table 6.1). Refer to table 6.2 for media preparations and reagents used for experiments in this chapter.

6.2.1.2 SV2A Expression Analysis Using iCell Neurons

iCell neurons were plated at 225,000 cells/well on PLO and Matrigel coated 24-well plates (procedure described previously) and grown for 7 and 14 days. Cells were collected in 60µL of RIPA buffer with protease inhibitors (3 wells per sample) and stored at -80◦C until Western Blot Analysis. (see table 6.2).

6.2.1.3 Control SV2A and SV2C Standards Analysis

Peptides for both SV2A and SV2C were purchased from Synaptic Systems to enable optimisation of the Western Blot analysis specifically for the detection of SV2 proteins (see table 6.1). The peptides were heated to 95˚C for 5 minutes before being added to a 4-20% TGX gel, analysing the peptides by loading 5µg/well (in 20µL of PBS). The peptides were migrated through the gel following the Western Blot protocol and transferred to a nitrocellulose membrane for probing with antibodies. Primary antibodies were added to the blot at a concentrations of 1:500, 1:750, 1:1000 and 1:1500 to attempt to determine

optimal concentration and secondary antibody was used at 1:10000 for each experiment.

Antibody	Supplier	Product Code
SV2A Rabbit Polyclonal	Synaptic Systems	#119 002
SV2C Rabbit Polyclonal		#119 202
SV2A Control Peptide		#119 0P
SV2C Control Peptide		#119 2P
Secondary HRP Goat Anti-rabbit IgG (H+L)	Jackson ImmunoResearch	#111-035-144

Table 6.1: Antibodies and Controls Used in SV2 Analysis.

6.2.2 Addition of Gt1b to Culture

SH-SY5Y cells were expanded for 7 days after thawing before plating at 250,000 cells/25cm² flasks. Three flasks per experimental condition were prepared; 30U/mL toxin, 0U buffer control, Gt1b 30U/mL toxin and Gt1b 0U buffer control. The Gt1b was added 24 hours before toxin treatment at a concentration of 50µg/mL, and the cells were incubated with toxin for 48 hours. Images of the cells were captured at the 48-hour time point, after addition of Gt1b and toxin, and cell density was analysed. The cells were then lysed in RIPA buffer with protease inhibitors and stored at -80˚C before Western-blot analysis.

iCell neurons were also grown for 7 days, plating at 225,000 cells/well on a 24-well plate. Gt1b was added 24 hours before toxin treatment at a concentration of 50µg/mL.

Solution/Media	Components	Volume/Concentration
BE(2)-C and SH-SY5Y Maintenance Media (40mL)	EMEM (EBSS) Hams F12 Non-Essential Amino Acids Glutamine Heat-inactivated FBS Penicillin/Streptomycin	16.6mL 16.6mL 400µL 400µL 5.6mL 400µL
RA Media (500µL)	Maintenance Media 50µM RA Stock Solution	490µL 10µL
DMSO Control (RA) (500µL)	Maintenance Media DMSO	490µL 10µL
Toxin Media 30U/mL (500µl)	Maintenance Media Toxin	470µL 30µL
Toxin Buffer Media 0U/mL (500µL)	Maintenance Media Buffer	470µL 30µL
Matrigel Coating Solution (8mL)	Matrigel Stock Solution iCell Maintenance Media	62.7µL 8mL
iCell Maintenance Media (100mL)	Supplied Media Supplied Supplement Penicillin/Streptomycin	97mL 2mL 1mL

Table 6.2: Medias and Reagents Used in Analysis in Chapter 6: Receptor Expression.

6.3 Results

6.3.1 Analysis of SV2 Expression with Western Blot- Neuroblastoma

Western Blot was used to analyse the expression of toxin receptor protein SV2; isoforms SV2A and SV2C were analysed firstly in neuroblastoma cell samples. SV2A was selected as it is recorded as being an abundant protein in the brain and SV2C as it is a specific target receptor for Botulinum toxin type-A. The results from analysis of expression of both isoforms in BE(2)-C differentiated cell samples are shown in the figures below.

6.3.1.1 SV2A

Three replicate analyses of BE(2)-C and SH-SY5Y RA differentiated cell samples were collected and each analysed three times with Western Blot; all with the same results. A blot for the analysis of BE(2)-C cells is shown in figure 6.6 below. Toxin treated samples, with a comparable control, were also analysed for expression of SV2A, with the same results. SV2A could not be detected in BE(2)-C cells. Similar results were shown for the SH-SY5Y cells for SV2A analysis; each blot unable to detect SV2A in the samples.

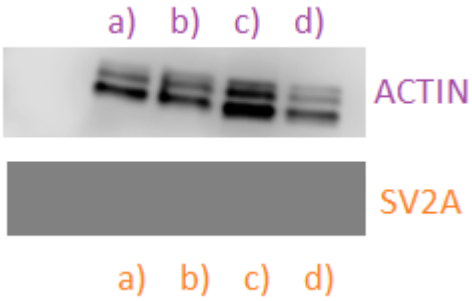


Figure 6.6: Western Blot Analysis of SV2A on BE(2)-C Cell Samples. *(a) SV2A Retinoic acid differentiated cells (a) Actin (b) SV2A DMSO control for RA sample (b) Actin (c) SV2A Toxin treated 30U/mL (c) Actin (d) SV2A Buffer control 0U/mL (d) Actin.*

6.3.1.2 SV2C

The SV2C expression analysis provided inconclusive results. The antibodies were trialled at three different concentrations (1:100, 1:1000 and 1:10000), each with no detection of SV2C bands. The protein band is expected to be visible at 95kDa, and figure 6.7 indicates the detection of a possible band. To potentially improve results and optimisation of the detection methodology, reference peptides for SV2C and SV2A were analysed opposed to further analysis using neuroblastoma cells. This would confirm the specificity of the antibodies, optimal concentration to yield results or highlight the lack of detection is due to the minimal expression of the SV2 receptors in the cell lines.

Figure 6.7: SV2C Western Blot Analysis of SV2C on BE(2)-C Cell Samples. *1:100 concentration of antibody. SV2C at 95kDa, arrow shows possible band.*

6.3.2 Analysis of SV2 Expression with Western Blot – iCell Neurons

The detection of SV2A and SV2C was not successful with the neuroblastoma cell lines, but this could be since the protein receptor is not expressed in the BE(2)-C or SH-SY5Y cells at all. It was the intention to optimise antibody concentrations with the 'cheaper', passage-able cell lines but as detection was not conceivable, analysis of SV2A expression was carried out using iCell neurons which have been shown to abundantly express SV2A (see figure 6.5).

The blot shown in figure 6.8 below was obtained by analysing four samples of untreated iCell neurons collected on day 7 and day 14 of culture.

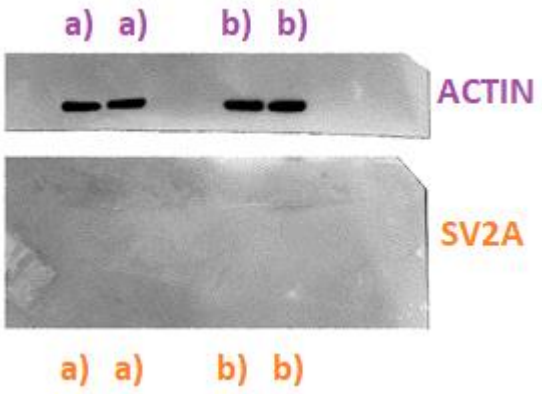


Figure 6.8: Western Blot Analysis of SV2A Expression in iCell Neurons. *(a) SV2A iCells day 7 of culture (a) Actin (b) SV2A iCells day 14 of culture (b) Actin. Antibody used at a concentration of 1:1000.*

6.3.3 Analysis of SV2 Controls

6.3.3.1 SV2A Controls

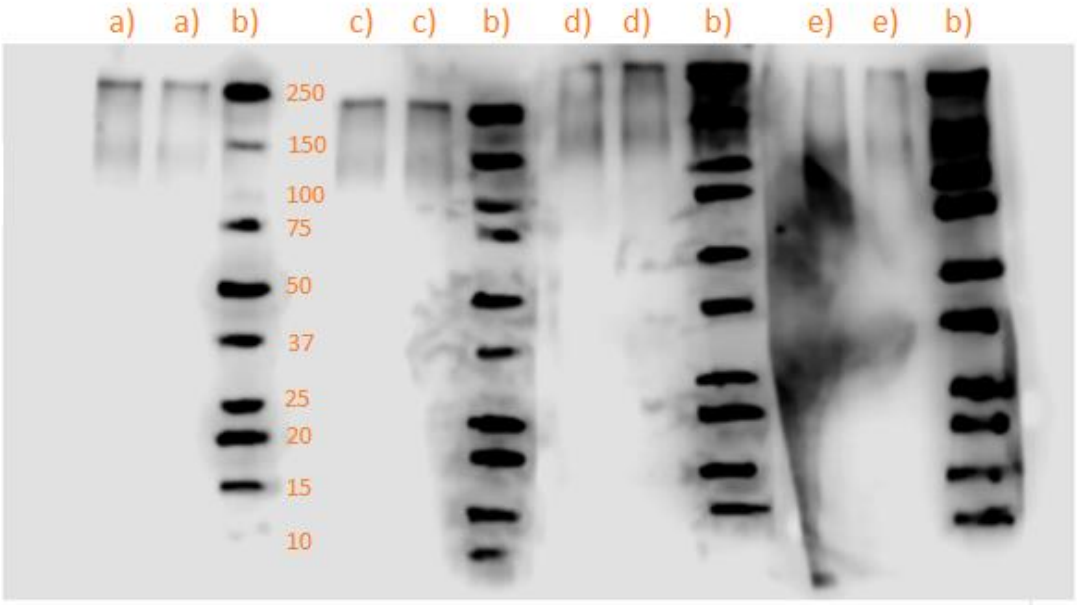


Figure 6.9: SV2A Control Peptide Western Blot Analysis Results. *4 blots shown in same image with differing primary antibody concentrations* ***(a)*** *1:1000* ***(b)*** *Molecular Weight Marker* ***(c)*** *1:1500* ***(d)*** *1:750* ***(e)*** *1:500. 5µg/20µL/well of peptide. Molecular Weight Marker labelled in kDa.*

SV2A is expected to sit at 82-92kDa and as the four blots in figure 6.9 above show, no detectable bands at this molecular weight are visible. There are bands detected at approximately 250kDa, which would suggest an issue with the migration of the peptides and aggregation of SV2A in the wells of the gel. The same results are shown for each of the four blots with varying concentrations of primary antibody, rendering optimisation of antibody concentration incomplete at this stage.

6.3.4 Control SV2C Peptides

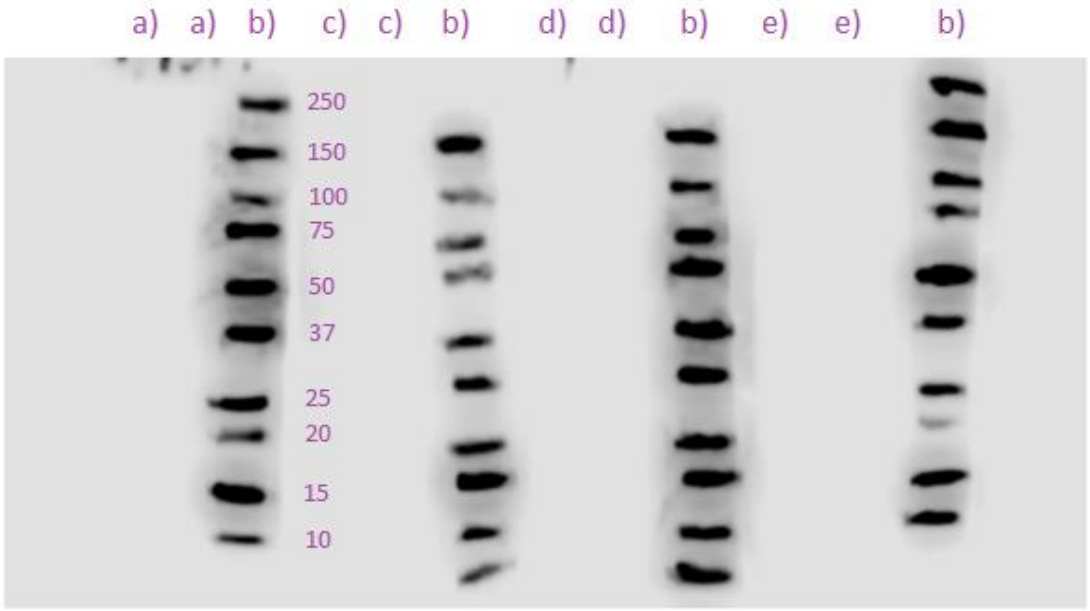


Figure 6.10: SV2C Control Peptide Western Blot Analysis Results. *4 blots shown in same image with differing primary antibody concentrations* ***(a)*** *1:1000* ***(b)*** *Molecular Weight Marker* ***(c)*** *1:1500* ***(d)*** *1:750* ***(e)*** *1:500. 5µg/20µL/well of peptide. Molecular Weight Marker labelled in kDa.*

As shown in figure 6.10, the four analysed concentrations of primary antibody for the SV2C analysis did not provide any detection of the control peptide thus we are unable to specify the optimum concentration to enable detection of SV2C in cell samples.

6.3.5 Adding Gt1b Ganglioside to Culture to Improve Sensitivity

Gt1b added to culture was first trialled on the SH-SY5Y neuroblastoma cell lines to ensure that the reagent did not have any detrimental effect on the cells when added to the media, before incubation on the more expensive iCell neurons. Cell density was evaluated, using trypan blue cell count, on addition of Gt1b to analyse cell viability in culture and the results are shown in figures 6.11 and 6.12 below.

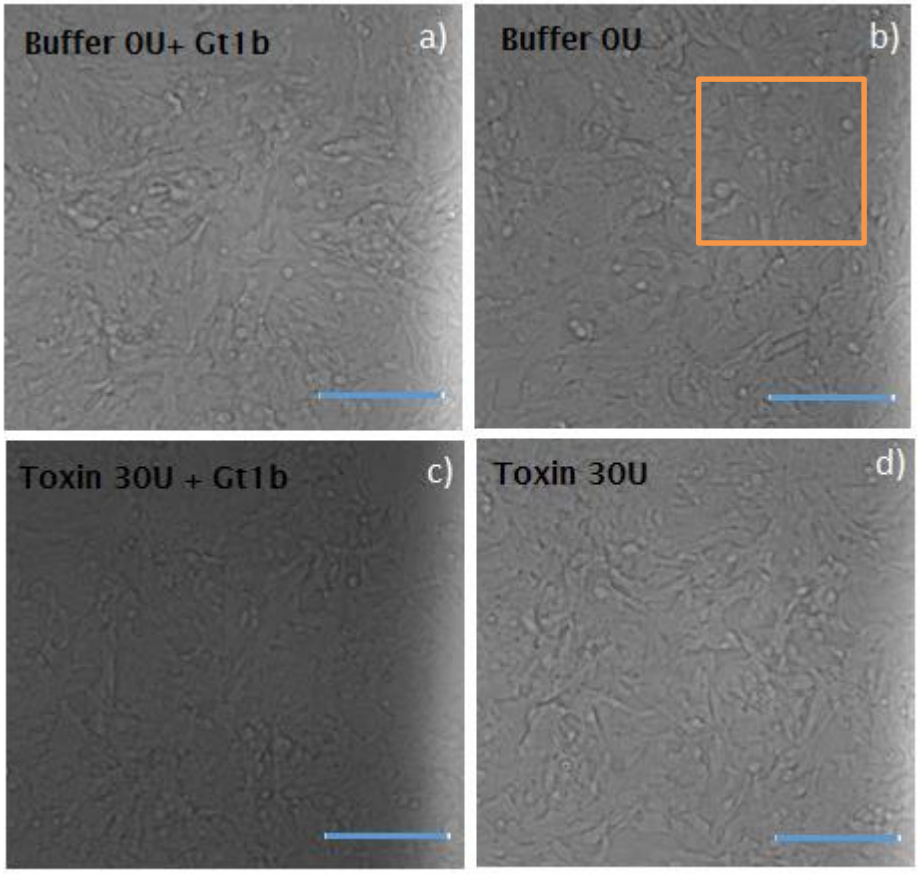


Figure 6.11: Images Captured of SH-SY5Y for Qualitative Cell Density Analysis. *Cells were counted within the box show on figure 6.11b in three sections of the image, and this was repeated for three images per concentration. Scale bar 100µm.*

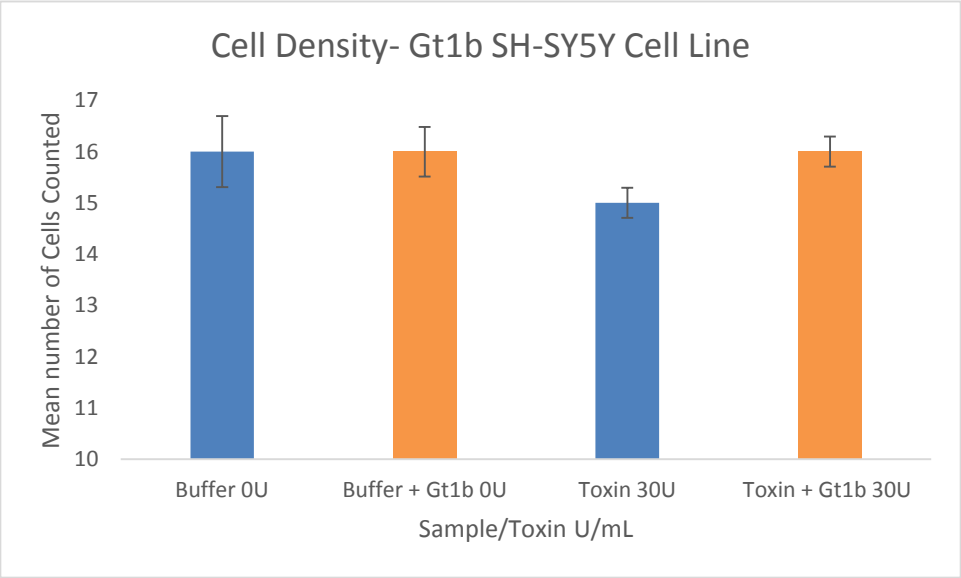


***Figure 6.12: Cell Density Quantification**: Bar chart representing the average number of cells per image in the 4 different conditions. Bars represent means and error bars represent the standard error of the mean. N=3 independent experiments, with 3 pictures analysed per treatment. p=0.7189 with two-way ANOVA test which shows the differences in cell density is not significant.*

The figure 6.12 bar chart above shows that the average number of cells does not significantly differ in each experimental condition: 0U/mL buffer, 0U/mL buffer with Gt1b, 30U/mL toxin and 30U/mL toxin with Gt1b. There is no statistical significance in the cell density across the four experimental conditions and this result confirms that the addition of Gt1b gangliosides does not affect cell number and it can be added to culture with no risk.

The same stock of Gt1b was then added to iCells on day 6 of growth. It was intended that the cells be incubated in Gt1b for 24-hours before toxin incubation, so toxin testing would commence of day 7. The same concentration of ganglioside was added to the culture as for the SH-SY5Y cells but when the cells were observed before the media change, it was apparent that a large population of the adherent cells treated with Gt1b had become detached from the plate. This is concerning as the iCells have been consistently robust in their culture throughout the project and indicated a problem with the incubation with

Gt1b. The wells set aside for the toxin and control experiments without Gt1b remained adhered to the plate, but on day 8 of culture it was noticed that there was a mild contamination. This could suggest an issue with the vial of iCells, an issue with media used or a contamination in the laboratory and incubator that had spread to the experimental plates.

6.4 Discussion

As discussed in the introduction to this chapter, SV2 is suggested to be an important receptor in the binding of BoNT-A, allowing toxin entry into the cell and its subsequent paralytic effect. We aimed to focus on the detection of two of the three isoforms; SV2A and SV2C, as these are the most abundant (SV2A) and most documented to have proven interaction with BoNT-A (SV2C). The paper by Whitemarsh (et al, 2012) had previously shown that SV2A was detectable in iCell neuron samples, clearly expressed from day 4 of culture. Their experiments did not show an abundance of SV2B which led to the dismissal of analysis for this isoform and the detection of SV2C was also not present in the iCell neurons; results which are parallel to the results of this project. Although it cannot be strictly determined that there is no presence of SV2C in the cells, as optimisation of the antibody was not achieved, literature suggests that it would be unlikely to detect this isoform in the iCell neurons anyway. The lack of expression or detection of SV2A is more troublesome, with the literature results clearly displaying Western Blot analysis of SV2A and our results do not reflect comparable results. This could be for many reasons, with the most probable being that the cell density and subsequent protein concentration used in our analysis is potentially a lot less than that used in the published literature (see figure 6.13 where published blot is shown again for clarification).

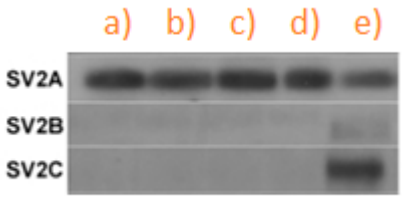


Figure 6.13: Western Blot from Whitemarsh (et al, 2012). *Adapted to only show discussed proteins SV2A, SV2B and SV2C.* ***(a)*** *4 days* ***(b)*** *7 days* ***(c)*** *14 days* ***(d)*** *21 days* ***(e)*** *Rat spinal cord cells.*

Gt1b Ganglioside in Culture: It is well documented in the botulinum toxin research field that the presence of receptors, specifically SV2 and ganglioside Gt1b is necessary for type-A binding to neuronal cells (Schengrund, DasGupta et al. 1991, Kozaki, Kamata et al. 1998, Yowler and Schengrund 2004). The results in figure 6.12 demonstrated that the Gt1b ganglioside, incubated with the culture at a concentration of 50µg/mL did not have any detrimental effect on the neuroblastoma cells but when this concentration was used to analyse the hiPSC cell line selected it caused cell detachment and death. If the attribution of Gt1b to culture did not potentially infringe on patented methodologies and had support from the client sponsor, the concentration would have been reviewed, or a new stock purchased to analyse the effects of Gt1b. At the completion of this project at the University, the timescale did not permit for continued analysis at this stage.

6.5 Conclusions

Chapter 6 did not render any conclusive or positive results with regard to analysis of SV2 receptors but literature reviews have confirmed that these receptors are expressed by the selected cell line, iCell neurons, thus confirming our selection of cells sensitive to BoNT-A and suitable for use in a potency assay for the pharmaceutical testing of this product. It was unfortunate that there was not more time to optimise these experiments to confirm this observation for this project, but original time lines were affected by the extensive optimisation of the Western Blot method that was required and the assignment of more time for the screening of suitable detection methods, as discussed in chapter 7.

The influence of adding Gt1b to culture media to improve toxin sensitivity was also a neglected element of research, strongly influenced by the price of the iCell neurons, time assigned to other tasks and guidance from an important client sponsor, who deemed the use of the ganglioside unnecessary at this time.

Future work is required on the development of a cell-based assay for the potency testing of Botulinum toxin type-A and is discussed in further detail in Chapter 8.

Chapter 7: Detection Methods

7.1 Introduction

As defined in Chapter 2, the second project aim is to select and optimise a detection method for the quantification of Botulinum toxin type-A activity and thus determining its potency. Chapters 3-6 have determined the selection of the iCell neurons for use in the cell-based assay, based on their consistent expression of SNAP-25, their sensitivity to toxin and the reproducibility of the results between each experiment. With the first aim of the project completed, it is essential that the selected detection method is both compatible with the selected cell line and robust in its methodology, allowing for the adaptation of the detection method to ensure ICH guidelines and GMP compliance.

Three detection methods were selected for screening; Western Blot, Mass Spectrometry and Microchip Electrophoresis. Each detection method has appealing attributes for use in a cell-based assay, outlined in table 7.1 below.

Detection Method	Benefits/Reason for Selection
Western Blot	• Previous use reported in literature • Antibody detection is sensitive • Detects both cleaved and uncleaved SNAP-25
Mass Spectrometry	• Sensitive method • Non-antibody approach- novel • Previously used to determine BoNT serotypes
Microchip Electrophoresis	• Uses fluorescence- novel • Small sample volume required • High-throughput

Table 7.1: Potential detection methods for a cell-based assay. *These methods were preliminarily outlined for the potential to be sensitive and adaptable for detection of botulinum toxin type-A activity.*

The initial cell screening took place with the use of Western Blot for the detection of toxin activity and expression of SNAP-25. It was selected because the method is used predominantly in literature for the screening of cell lines for sensitivity to toxin and antibody detection is a sensitive method. It has been shown to detect both cleaved and uncleaved SNAP-25 as a result of toxin activity, shown in literature and in the results from Chapters 4 and 5, and proved essential in the screening of the cell lines for defined sensitivity criteria.

Chapter 2 defined specifications and requirements for the approach to screening the detection methods and analysing its sensitivity, and stop/ no-go points that were applied to each of the selected methods to ensure optimum allocation of time on each method. The criteria 1-4 are defined in the summarising table below, (table 7.2) and each is an essential requirement of the detection method. Criteria 1 is the first approach to analysing sensitivity; the detection of the protein of interest is of upmost importance. The detection method must be repeatable and reliable in its detection of SNAP-25 expression (criteria 3 and 4) and the distinction between cleaved and uncleaved is essential for the quantification of potency and determination of toxin activity.

It is regarded as an immediate stop/no-go point if the detection method does not successfully detect whole SNAP-25. Continuing analysis of this detection method would not be beneficial as this detection is an essential element to the design of the required cell-based assay. Similarly, if a selected method detects SNAP-25 but distinction between cleaved and uncleaved is not achievable, this detection method would be discarded for suitability. These requirements are summarised in table 7.2 below.

Criteria	Specifications/Requirements	Stop/no-go points- apply to all methods
1	Detection of Whole SNAP-25	• Detection of whole SNAP-25 not possible • Distinction between cleaved and uncleaved SNAP-25 not achieved • GMP compliant method/quantification not feasible.
2	Distinction between cleaved and uncleaved SNAP-25	
3	Repeatable and reliable results with the selected cell line	
4	Reliable quantification and determination of potency	

Table 7.2: Defined criteria and stop/no-go points in the selection of a detection method. *Four defining criteria in the approach to detection method screening with associated stop/no-go points, which apply successively to all selected detection methods.*

When analysing a detection methods suitability, it is also necessary to consider the regulatory submission requirements of the cell-based assay as a whole. Accuracy, precision and robustness are but a few of the requirements to ensure a replacement for the mouse bioassay is a compatible candidate; producing comparable results to that of the *in vivo* test. The criteria, represented in Chapter 2, and in table 7.3 below for clarification, are to be considered in the selection of a detection method, which must be capable of generating results as sensitive as those captured from the mouse assay. The most influential criteria are that of precision and robustness. These incorporate the necessity of repeatable results and a methodology for the testing of such toxic products must be robust in its results, to ensure public safety. All elements of the detection procedure should be repeatable, reliable, not influenced by variables, such as laboratory settings, the users or susceptible to high likelihood of human error.

Criteria	Definition	
Accuracy	The closeness of agreement between the value which is accepted as a true or reference value and the value found	
Precision	Closeness of agreement between series of measurements obtained from multiple sampling of the same sample under same conditions.	
	Repeatability	Under same operating conditions over short interval
	Intermediate Precision	Within-laboratory variations; different days, analysts, equipment.
	Reproducibility	Precisions between laboratories (collaborative)
Detection Limit	The lowest amount of analyte detectable in a sample	
Quantitation Limit	The lowest amount of analyte quantifiable with suitable precision and accuracy.	
Linearity	Ability (within a given range) to obtain test results which are directly proportional to the concentration of analyte	
Range	Interval between the upper and lower concentration of analyte for which it has been demonstrated that there is a suitable level of accuracy, precision and linearity	
Robustness	Measure of its capacity to remain unaffected by small variations in method parameters; an indication of its reliability during normal usage	

Table 7.3: Criteria Definitions and Regulatory Expectations. *The ICH guidelines for the validation of analytical procedures (2005). Refer to chapter 2 section 2.5 also.*

7.1.1 SNAP-25 Reference Standards

When analysing the capabilities of a detection method the use of a standard reference is an essential tool in the assurance of a working methodology. This project focusses on the detection of SNAP-25, in both its whole (206 amino acids) and toxin cleaved (197 amino acids) forms, and a detection method used in the cell-based assay for the potency testing of Botulinum toxin must detect and distinguish between these two forms. Recombinant standard reference proteins

for whole SNAP-25 and the long portion of cleaved SNAP-25 were produced, outsourced to Toxogen. These standards were used in Western Blot analysis to confirm the detection capabilities of the methodology, and were a useful tool in the confirmation of working Western Blots when analysed alongside protein samples for the presence of SNAP-25 for the cell screening experiments.

7.1.2 Western Blot Detection Method

Western Blot is widely used for the detection of specific proteins in cell lysate or tissue homogenate extracts. This analytical technique uses gel electrophoresis, separating proteins by molecular weight (Mahmood and Yang 2012). The proteins are then transferred to a membrane, nitrocellulose or PVDF, allowing the proteins to be incubated with antibodies specific to the target protein; anti-SNAP-25 antibodies in the case of this project. Unbound antibody is then washed off, leaving only the ones bound to the protein of interest, and the antibodies are detected for quantification with enhanced chemiluminescence onto photo film or detected with an ECL scanner. The signal intensity of the band corresponds to the amount of protein present in the sample, and detecting a house-keeping protein, such as Beta-Actin, alongside as a standard control, allows the comparison of intensity for quantification (Mahmood and Yang 2012).

The standard technique for Western Blot analysis is outlined below; for the detection of SNAP-25 this protocol is slightly modified as described in the methods section.

7.1.2.1 Sample Preparation

Protein extraction intends to collect all proteins in the cell cytosol and membrane, performed at a cold temperature and on ice with protease inhibitors to prevent protein denaturing. For preparing tissue samples sonication may be necessary to ensure all cells are lysed. Analysis of the protein concentration is necessary to determine quantity loaded onto the gel and allows an equivalent comparison. The sample is then diluted in loading buffer containing glycerol to ensure ‘smooth’ migration of the proteins through the gel and a tracking dye to assess separation progression. Heating the sample is important to denature the

higher order protein structure whilst retaining sulphide bonds (Mahmood and Yang 2012) ensuring that the negative charge of amino acids is not neutralised.

7.1.2.2 Gel Electrophoresis

When loaded onto the gel, proteins migrate towards the positive electrode when a voltage is applied, ensuring separation of the proteins through the gel according to their size. The loading buffer with dye is a clear indication of the migration process; and the voltage should cease when the proteins of interest reach the central or lower part of the gel. Proteins will stay in the gel at corresponding weight markers, providing separation for antibody detection.

7.1.2.3 Blotting

After separation, the proteins are transferred to a membrane, using an electric field perpendicular to the surface of the gel, encouraging the movement of the proteins from the gel to the membrane. When placing the membrane between the gel and the positive electrode it is important to ensure migration of the proteins in the correct direction; onto the membrane rather than a protective filter paper.

7.1.2.4 Antibody Incubation

Blocking unspecific binding sites on the membrane is an important step to prevent unspecific signal, usually using a solution of dried skimmed milk powder diluted in tris-buffered saline with tween (TBST). This reduces the background in the detection providing clearer blots and results for quantification. The blot is then incubated in the primary antibody solution overnight at 4˚C. Washing of the blot after primary incubation is important to minimise the background interference and to ensure the removal of unbound primary antibody. Addition of the secondary antibody, usually labelled with horseradish peroxidase (HRP) for a further 30 minutes amplifies the signal for detection.

7.1.2.5 Chemiluminescence

The use of a horseradish peroxidase (HRP) labelled antibody enables the oxidation of luminol and the reaction is accompanied by the emission of light. It is possible to enhance this light emission, referred to as enhanced chemiluminescence (ECL), using a HRP-labelled antibody which catalyses the ECL substrate producing an excited carbonyl, which emits light. The membrane, once bound with the secondary HRP-antibody, is submerged in ECL solution (Amersham) for 30 seconds before exposure to film or CCD imaging systems.

7.1.2.6 Quantification

Data produced by Western Blot is typically considered semi-quantitative as it provides a relative comparison of protein levels but not an absolute measure of quantity (Mahmood and Yang 2012). Variations in loading and transfer rates over the several lanes on a gel are an issue and the signal generated by detection is not considered linear.

7.1.2.7 Summary

Western Blot is susceptible to many problems and errors; faint bands or no signal, high background which will affect the intensity quantification or unexpected detection of bands due to low specificity of the antibodies. These extra bands can be due to different forms of the protein or degradation of the protein of interest, affecting the molecular weight of the protein; producing several bands for the same protein or high voltage during the migration through the gel. These problems and variability of results have contributed to the decision of using Western Blot as a detection method for the screening of cell line sensitivity to botulinum neurotoxin but not as the final detection method for a GMP compliant cell-based assay.

7.1.3 Mass Spectrometry Detection Method

In proteomics, LC-MS (liquid chromatography-mass spectrometry), or also referred to as HPLC-MS (high performance), is used as an analytical chemistry technique for the identification and mass analysis of proteins (Pitt 2009). It works by converting analyte materials to a ionised state and analyses based on the mass to charge (m/z) ratio of the ions and fragmented ions. Electrospray ionisation is the first step; liquid sample is pumped through a capillary forming a fine spray, or aerosol, of charged droplets. Electrical energy is used to assist the transfer of the ions from solution to gaseous phase for analysis with the mass spectrometer (Ho, Lam et al. 2003). Droplets leave the electrospray tip and pass down a pressure and potential gradient towards the analyser, where they are analysed by molecular mass and ion intensity.

Mass Spectrometry is predominantly used to detect, quantify and differentiate between several serotypes of botulinum neurotoxin (Barr, Moura et al. 2005). However, it has yet to be proven as to whether a cell-based assay can use mass spectrometry as the method for detection of whole SNAP-25 or of SNAP-25 cleavage as a measure of toxin activity. Compared to the detection of presence of toxin in samples for which it has been used previously, the detection of cleavage of SNAP-25 may prove more challenging and complex in its design of mass spectrometry detection assays.

It is a reliable and robust method, and will be trialled as a method using iCell neuron cell samples treated with toxin. The project intends to look at detecting whole SNAP-25 (206 amino acids) alongside the long (197 amino acids) cleaved peptide and the small fragment 9 amino acid peptide as means of identifying cleavage of SNAP-25 as a result of toxin exposure. The whereabouts of this small 9 amino acid peptide is unknown after toxin exposure, it is undetermined as to whether it can be detected. This would be a novel approach to the detection of toxin activity in an in vitro assay.

Optimising the protocol for the mass spectrometry analysis of SNAP-25 required several adjustments to the experimental methodologies. Analysis on iCell neurons and neuroblastoma cell lines are described below in the methodology.

7.1.4 Microchip Electrophoresis Detection Method

7.1.4.1 Introduction

Microchip electrophoresis provides protein separation much like a western blot by separating according to size on a gel, however the system is high throughput and needs small quantities of sample in comparison to the standard Western Blot technique. It will be interesting to pursue this detection method for the detection of botulinum toxin activity to determine its sensitivity as a method for a cell-based assay.

The 'Gelchip' has been designed by Professor Xize Niu at the University of Southampton Department of Engineering, and the technology was kindly available for our project analysis of detecting cleaved and uncleaved SNAP-25. Droplet-based microfluidics enables analysis of mulitple sample droplets in a high throughput approach. For general analysis, sample droplets are typically generated by a bioreactor or an upstream separation column and are injected into the separation channel for analysis. The chip utilises a novel approach; achieving droplet interfaced separation using a 'slipping' motion to push the droplets into the channels. This approach was designed to enable the separation of the entire volume of the droplet; quantifying biomolecules in the same sample. This was deemed suitable for our use as it would allow the potential distinction between cleaved and uncleaved SNAP-25 in the same sample droplet.

The samples are loaded onto the chip, and an electrical charge is applied that induces the migration of the proteins in the sample through a gel in SDS buffer. The proteins are then detected fluroescently, with the addition of a fluorescently tagged primary antibody, or a secondary antibody if amplification is needed for low abundance proteins and analysed under the microscope. Using these droplet-interfaced microchip electrophoresis techniques, Professor Niu and his collegues have developed a gelchip platform (figure 7.1) which allows fluid loading of multiple sample droplets into the separation channels (Hassan, Morgan et al. 2015). Our aim is to use this platform, permitting the ability to analyse mutliple samples at once in a short amount of time.

7.1.4.2 Application in Principle

The 3D schematic shown in figure 7.1 below was provided by the Engineering department from the University of Southampton, and demonstrates the application of sample separation and use of the microchip technology.

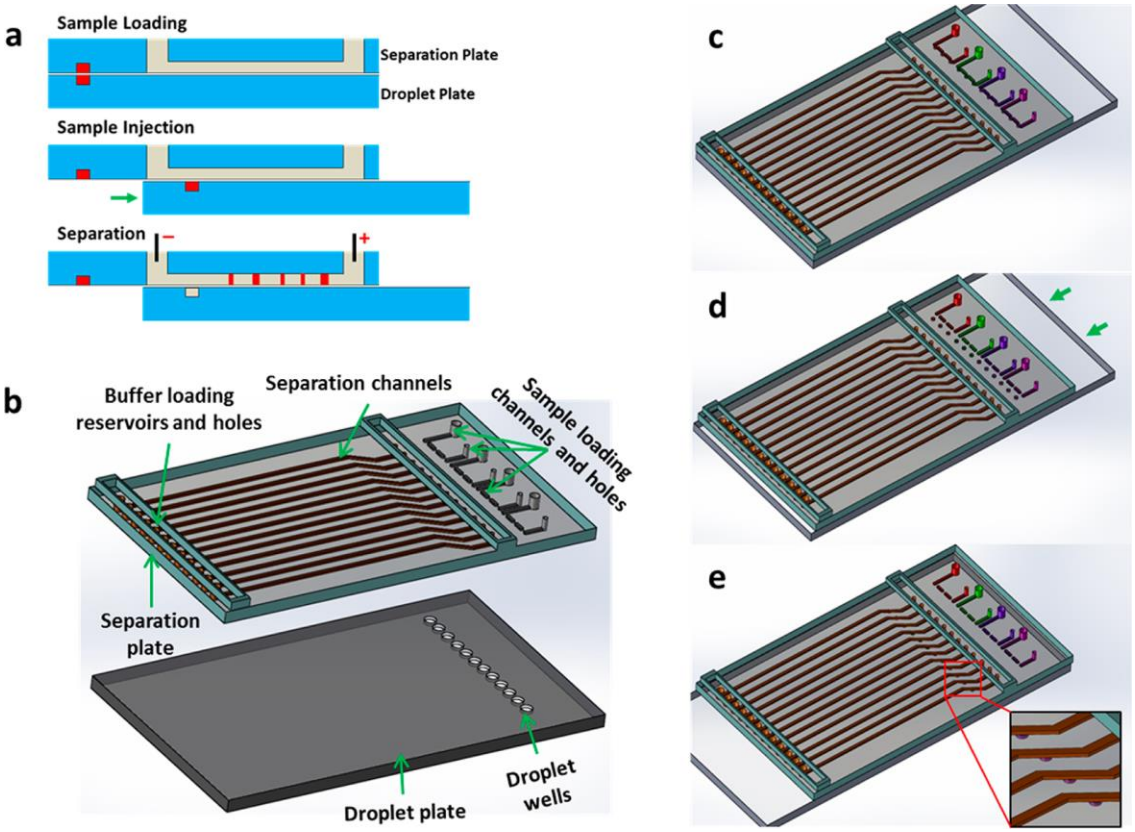


Figure 7.1: 3D schematic of the microchip application. (a) *droplet generation, injection and sample separation, arrow indicates movement direction of plate* ***(b)*** *separation and droplet plates* ***(c)*** *initial position of the chips after assembly of the component and loading of samples* ***(d)*** *the slipping bottom layer which generates droplets* ***(e)*** *injection of sample droplets into the separation channels. Image provided by Professor Xize Niu University of Southampton Department of Engineering.*

Figure 7.1 shows the assembly of the microchip and sample droplet generation, injection and separation with the direction in which to move the droplet plate marked with an arrow. Magnets ensure that the two plates are securely

assembled and 'slipping' the chip allows for generation of sample droplets that are aligned with the separation channels for analysis. Application of an electric current allows for the migration of the samples through the separation matrices in the channels and separated molecules are fluorescently detected for quantification.

Droplet generation is calibrated by loading fluorescein dye solution into the sample loading channels connected to the wells, shown in figure 7.2a below. When the chip is 'slipped' each sample produces three droplets shown in figure 7.2b and the intensity of the fluorescence of the droplets provides data surrounding the reprocucibility of droplet area, shown in figure 7.2c. Figure 7.2d shows droplets repositioned to align with the separation channels.

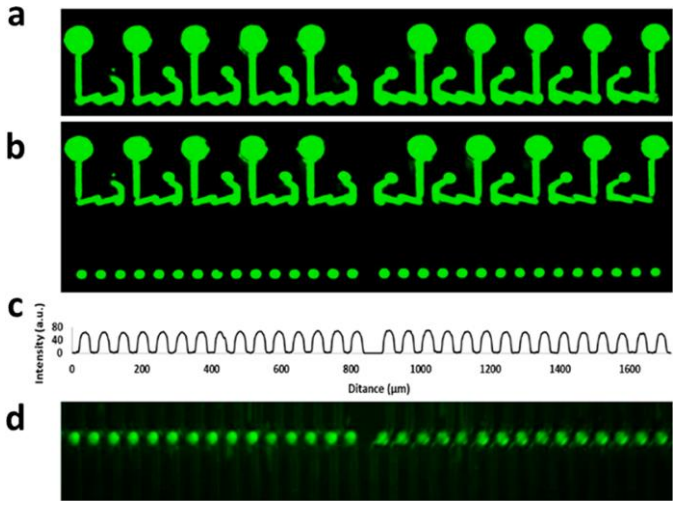


Figure 7.2: Droplet generation and injection for Microchip Electrophoresis. *Sample droplet generation is shown with the use of fluorescein dye.* ***(a)*** *dye is added to the wells* ***(b)*** *slipping the chip enables the generation of three replicate droplets* ***(c)*** *three droplets reproducibility is analysed* ***(d)*** *samples are positioned to enter the channels. Image provided by Professor Xize Niu University of Southampton Department of Engineering.*

7.2 Methods

7.2.1 Analysis of SNAP-25 Standards

Recombinant protein reference standards for whole (206 amino acids) and cleaved (197 amino acids) SNAP-25 have been purchased synthesised as controls for the detection method screening. Analysis of the specificity of these controls was undertaken; running a Western Blot using anti-SNAP-25 polyclonal antibody (Sigma #S9684) to detect SNAP-25 protein and a Coomassie blue staining for detection of all proteins in the standards.

7.2.1.1 Coomassie Staining Protocol

After migration of the standards through the gel via Western Blot, the gel is immersed in the staining solution (1 litre: 500mL methanol, 400mL ultrapure water, 100mL acetic acid, 2.5g coomassie brilliant blue R-250, filtered through chromatography paper to remove impurities) and then microwaved for 30 seconds. The gel is then placed on a rotating platform for 30 minutes or until the bands start to become visible. It is then rinsed in water several times to remove the staining solution and immerses in de-staining solution (1 litre: 785mL ultrapure water, 165mL ethanol, 50mL glacial acetic acid) and microwaved for a further 30 seconds. The gel is then left to rotate and shake until the background is clear and bands are revealed.

7.2.2 Western Blot Analysis

Samples were diluted 1:1 in LDS sample buffer (Life Technologies). The loading buffer indicates migration of the proteins through the gel. 25µl of each sample was loaded onto on 12% TGX Protean gels, with 1 well (10µl/well) of BioRad Dual Colour Markers; a molecular marker ladder for identifying protein bands (see Appendix C). The gel was loaded into the BioRad Tetra system, submerged in running buffer (see table 11) and run at 200V for 30 minutes; until the LDS sample buffer reached the base of the gel. The proteins are separated in the gel according to molecular weight and transferred to a nitrocellulose membrane

(blot) in transfer buffer (table 7.4). (See Appendix D for detailed protocol). The membrane is then blocked, in 20mL 5% milk powder (see table 7.4) for 30 minutes; this procedure blocks nonspecific binding sites on the membrane, contributing to clearer blots and more specific binding of the antibody to the protein of interest. To ensure the best possible chance of clearly detecting the low abundant SNAP-25 protein, it was decided that to separate both the actin and SNAP-25 analysis on the membrane would be beneficial to ensure cleaner blots and more specific detection. The membrane was cut in half at the 37kDa marker; clearly separating the SNAP-25 (25kDa) and Actin (42kDa) and each half was incubated in the corresponding antibodies. Following membrane blocking, the blots were incubated overnight in the primary antibodies; anti-SNAP rabbit polyclonal (Synaptic Systems #111 002) at a concentration of 1:1000 (appendix A) and anti-Beta-Actin rabbit polyclonal (Synaptic Systems #111 023) at a concentration of 1:20000. The membranes were then washed in 1x TBS three times before addition of the secondary antibody. The blots were incubated in HRP-anti-rabbit, 1:20,000 in 5% milk solution for 1 hour (appendix A). As both primary antibodies are rabbit polyclonal the two blots are incubated in the same secondary antibody. Results were generated, revealing protein bands on film with enhanced chemiluminescence (ECL) (see appendix E for protocol). Quantification of the intensity of the protein bands was performed using ImageJ software (appendix F).

Solution	Components		Quantity
Running Buffer	BioRad SDS 10x buffer Distilled water		100mL 900mL
Transfer Buffer	TRIS Glycine Methanol Distilled Water		3.03g 14.4g 200mL 800mL
TBS Buffer 10x	TRIS NaCl Distilled water	Adjust pH to 7.6 adding hydrochloric acid	61g 90g 1 litre
Blocking Buffer	Dried milk 1 x TBS		1g 20mL
Antibody solution	Dried milk 1x TBS		2.5g 50mL

Table 7.4: Western Blot Reagents used for detection of Beta-Actin and SNAP-25.

7.2.3 Western Blot Optimisation for SNAP-25 Cleavage Detection

The methodology required optimisation to ensure the successful detection of both cleaved and uncleaved SNAP-25, as discussed in Chapter 5. Adaptations included using 4-20% gradient gels and allowing migration of the samples through the gel for an increased time to ensure separation of the 25kDa and 24kDa proteins. Samples were collected as previously described (also see Chapter 3), diluted 1:1 in LDS sample buffer (Life Technologies) and heated to 95˚C in a heating block for 5 minutes. 20µl of each sample was loaded into 4-20% gradient gels (BioRad), and migration was initiated, at 200v, for 50 minutes. The proteins were then transferred to a nitrocellulose membrane, incubated in primary antibody overnight at 4˚C. Several antibodies for SNAP-25 were used alongside the antibody for the housekeeping protein Beta-Actin, and optimised. See Appendix A for antibodies, providers and concentrations.

Previous experiments discussed in the succeeding chapters indicated that the optimum concentration of primary SNAP-25 antibody (Synaptic Systems #111 011) was 1:1000; the concentrations were repeatedly optimised to ensure detection of SNAP-25, 1:10,000 was the suppliers recommendation but did not provide detection of SNAP-25. The antibodies trialled with the Western Blot methodology are outlined in table 7.5 below. Several successful blots have been produced with concentrations of 1:1000 using both of the monoclonal antibodies (see table 7.5). An ECL (enhanced chemiluminescence) revelation protocol using photographic film and developer to reveal the protein bands was optimised, to provide the best exposure times for the most quantifiable bands for analysis with ImageJ software. For the clearest detection of SNAP-25, the membrane was incubated in ECL for exactly 60 seconds, then photo film was exposed to the blot for 2 minutes; a much longer exposure time than suggested protocols for protein detection was necessary to detect minimal amounts of SNAP-25.

Antibody	Provider	Concentration
Rabbit anti-SNAP-25 polyclonal antibody	Synaptic Systems #111 002	1:1000
Mouse anti-SNAP-25 monoclonal antibody	Synaptic Systems #111 111	1:1000
Mouse anti-SNAP-25 monoclonal antibody	Synaptic Systems #111 011	1:1000
Rabbit anti-SNAP-25 polyclonal antibody	Sigma, #S9684	1:1000
Rabbit Beta-Actin (housekeeping protein) antibody	Synaptic Systems #251 003	1:20000

Table 7.5: Primary antibodies trialled in the optimisation of the Western Blot methodology. *The mouse monoclonal antibodies #111 111 and #111 011 were found to be the most successful for detection of SNAP-25 in our protocol.*

7.2.4 Mass Spectrometry

Mass spectrometry analysis was performed on the Thermo Scientific Orbitrap Fusion Tribrid mass spectrometer, at the University of Southampton, Centre of Proteomic Research.

7.2.4.1 iCell Neurons Mass Spectrometry Analysis

iCells were grown for 7 days, at 225,000 cells/well and treated with 30U/mL toxin for 48 hours and 4 wells were collected in 80µl of TEAB with protease inhibitors inhibitors (100mM Triethylammonium bicarbonate, 0.1% SDS) and stored at -80◦C. Samples were sonicated and washed through 30kD filters, then the supernatent was lypholised overnight. The samples were then prepared using C18 SpinTips sample preparation kit (Protea) which enables reversed phase solid phase extraction of peptide samples for mass spectrometry. It concentrates and desalts peptides in protein digests, which is suitable for cell extracts. The sample was then digested with trypsin, to define smaller detectable peptides and lypholised overnight before analysis on the mass spectrometer.

An adaptation was made when detection of SNAP-25 was unsuccessful; altering the filters to 10kD and repeating the previous experimental protocol. The small peptide of interest may be lost during the preparation filtration process. When cleaved with trypsin, the peptides of SNAP-25, are broken down into smaller more detectable peptides, cleaving peptides at carboxyl side of lysines (K) and arginines (R). The cleavage site of botulinum toxin is an arginine (R) also cleaved by trypsin. This interference, and possible misinterpretation of toxin cleavage and trypsin activity could be contributing to the lack of detection. Adapting the protocol to use Lys-C as opposed to trypsin was an attempt to reduce the risk of unsuccessful detection as a result of trypsin cleavage and peptide degradation. Lys-C cleaves proteins at the C-terminus of lysine amino acids avoiding the arginine cleaved by toxin and trypsin. Lys-C digestion was trialled using samples of 30U/mL Botox treated, 30U/mL toxin treated, 0U saline and 0U buffer controls, lysed in 100mM Triethylammonium bicarbonate and 0.1% SDS. As before these samples were prepared using C18 sample preparation kit (Protea). A reference peptide for the 9 amino acid peptide cleaved from the whole SNAP-

25 peptide was made and added to the sample solution. Detection of this sythetic peptide enables easier detection of the cleaved nine amino acid peptide.

7.2.4.2 High Cell Density Analysis Experiments

It was apparent that the low protein concentration of the iCell neuron samples was contributing to the lack of detection of SNAP-25 with LC-MS so high cell density samples were prepared to increase the likelihood of detecting the protein. The iCell neurons are too expensive to enable increasing the cell number per sample, so neuroblastoma cell lines samples were prepared.

Two samples from each cell line were prepared; BE(2)-C, IMR-32 and SH-SY5Y, with two different concentrations of SDS to see if this improved cell lysis and subsequent detection. BE(2)-C $5x10^6$ cells/sample, IMR-32 $3x10^6$ cells/sample and SH-SY5Y $1x10^6$ cells/sample were lysed in either 100mM TEAB, 0.1% SDS as used previously, or 100mM TEAB, 4% SDS. Samples were sonicated before determining the protein concentration.

7.2.4.2.1 Protein Concentration Analysis

Total protein concentration of each of the cell samples was determined before mass spectrometry analysis, to ensure the selection of the highest protein samples for analysis; increasing the likelihood of detecting SNAP-25. Following sonication of the cell lysate, samples were centrifuged at 300 *xg* for 20 minutes to decrease the bubbles created by the sonication process. 5µl of the cell sample was diluted 1:2 in TEAB with 4% or 0.1% SDS (sample dependant) and vortexed for 10 seconds. 2µL of each sample was pipetted onto a card, which is inserted into the detector machine, with a maximum of 4 samples per reading. Protein was analysed on a Direct Detect Concentrator (Merck Millipore) using the NIST BSA AM2 method, which is specifically for low protein concentration samples and deemed the most reliable analysis.

7.2.4.2.2 FASP Sample Preparation

Two samples with the highest protein concentration were then prepared for analysis with the FASP protocol and OFFGEL Fractionation.

BE(2)-C in TEAB, 4% SDS and IMR-32 in TEAB, 4% SDS provided the highest protein concentrations and were prepared for analysis using the FASP kit. This allows solubilisation of protein samples in SDS and digestion with trypsin. The samples were mixed with urea and digested with trypsin overnight. The samples were then cleaned with the C18 kit as described before and stored at -20˚C before OFFGEL fractionation.

7.2.4.2.3 OFFGEL Fractionation

Peptide OFFGEL stock solution was prepared (OFFGEL Buffer- GE Healthcare and Glycerol) and was added to each of the samples. IPG strips with gel one side were placed into the fractionation tray, with electrodes attached either end. The prepared sample pipettes onto the gel strip and the fractionator run overnight.

Solution	Components	Concentration
(TEAB)	Triethylammonium bicarbonate SDS	100mM 0.1%

Table 7.6: Composition of TEAB Buffer for Mass Spectrometry Analysis

7.2.5 MicroChip Electrophoresis

The 'Gelchip' microchip platform is based on droplet-interfaced microchip electrophoresis (MCE) techniques and works by efficient interaction between a droplet well plate and a preloaded gel plate with separation channels to allow loading of multiple sample droplets into the separation channels (Hassan, Morgan et al. 2015).

7.2.5.1 Protocol

The chip is assembled by clamping the separation and droplet plates together using magnets on opposites to ensure tight contact throughout use and fluorinated oil (FC-40) is added to the interface to seal the microchannels and minimise sample sticking. This oil lubricates the two plates and reduces surface friction when moving the plates. Separation matrices are added to the channels before the addition of sample; adding the matrices into the 0.8mm diameter inlet holes with a peristaltic pump at a flow rate of 3μL/min. The sample is loaded into the loading channels and the separation buffer (TBE/Tris-Ches/Gel-exact composition not disclosed) is loaded into the two buffer reservoirs, connecting the separation channels. 0.5mm diameter platinum wire electrodes are placed in each reservoir which provides an electric field to the parallel channels. Before application of an electrical current, the separation plate is moved by a micrometre which generates droplets in the separation plate which then become aligned with the separation channels. Application of an electric field across the reservoirs initiates migration of the sample molecule through the chip and subsequently ensuring electrophoresis separation. Calibration of the sample loading was shown in figure 7.2 previously, which demonstrates an experiment in which fluorescein dye solution was loaded into the channels connected to the droplet wells. Sliding the clip produced three replicate droplets and fluorescent intensity profiles provided information on the reproducibility of the droplet area. The data output is shown in figure 7.3.

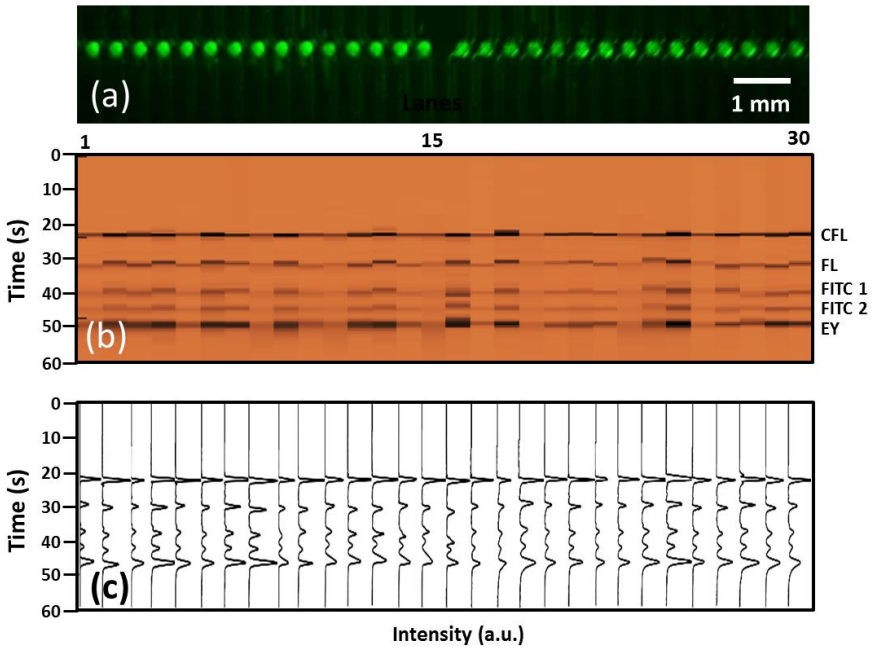


Figure 7.3: Nano-litre Droplets interfaced parallel separations *(provided by Professor Xize Niu, University of Southampton Department of Engineering)* ***(a)*** *Droplets introduced to separation channels.* ***(b)*** *Pseudo gel plot of sample mixture (Eosin Y, FITC 1, FITC 2, Fluorescein, 5-CarboxyFL).* ***(c)*** *Corresponding electropherograms. (Niu, Pereira et al. 2013).*

Molecules are detected using a fluorescent microscope and the recording of images with a CCD camera allows for the measurement of intensity of each band using ImageJ software.

7.2.5.2 Primary Antibody Fluorescent Labelling Experiments

7.2.5.2.1 Labelling Primary Antibody

The optimised antibody (Synaptic Systems #111 011) was labelled using the Lightning-Link® Rapid Atto488 kit purchased from Innova Biosciences. 1µl of LL-Rapid modifier reagent was added to 10µl of primary antibody and mixed gently. The kit contains a vial of Lightning-Link® Rapid mix and the mix of antibody and LL-Rapid modifier is added to the lyophilised product. Repeated, gentle pipetting ensures that the solution is mixed and properly diluted and the vial is left for 15 minutes at room temperature in darkness. 1µl of LL-Rapid quencher reagent was added to the suspension and incubated at room temperature for 4 minutes.

7.2.5.2.2 Analysis of Labelling Protocol

To analyse the success of the labelling protocol, the primary antibody was tested by Western blot. For analysis by Western Blot, iCell neurons (225,000 cells/well, 3 wells/sample) were collected in RIPA lysis buffer with protease inhibitors. The samples are diluted with LDS sample buffer 1:1.

Following the standardised protocol for the analysis of SNAP-25, iCell protein samples were migrated through a 4-20% gel and then transferred to a nitrocellulose membrane. The membrane was incubated with the labelled primary antibody at 4˚C overnight. Analysis was performed using a fluorescent microscope for detection of fluorescent signal.

7.2.5.3 Secondary Antibody Amplification Experiments

Following analysis of the primary antibody with Western Blot, a secondary amplification step was suggested for incorporation to the microchip methodology, making it comparable to the Western Blot protocol, given the previous issues surrounding the detection of SNAP-25. To ensure that SNAP-25 and cleaved SNAP-25 could be properly differentiated between using the microchip technology, the use of the reference standards was deemed the most

beneficial when optimising the method and concentrations. Addition of secondary fluorescent antibody to the reference standard samples was analysed to initiate optimisation of the microchip detection methodology. Samples were incubated overnight at 4 ˚C in the primary antibody (1:1000) and then secondary antibody (Alexa Fluor 488, 1:500) was added to the sample and it was incubated for 5 hours at room temperature in darkness.

The samples were then heated to 95◦C in a dry bath and LDS was added at a ratio of 1:2. The samples were heated a second time with the LDS before loading into the microchip. Three approaches to the methodology were experimented with; initially 7nL of sample was added to an assembled microchip utilising 2.5% PEO gel channels and a voltage of 170V/cm was applied. This was then adjusted to the addition of 100nL of sample in the 1mm wide and 0.4mm deep channels in the assembled microchip and application of 140V/cm voltage when using 2.5% PEO gel channels. An optimisation of the gel type was also trialled; using polyacrylamide gel (2.5%) and an application of 80V/cm voltage for migration of the samples. The separation and migration was recorded using the FITC filter on the microscope. The results for each condition are shown in the results section of this chapter. The detection chip should separate the samples into different fluorescent bands dependant on molecular weight; unbound primary and secondary antibodies and SNAP-25 (cleaved and uncleaved) tagged with the primary and secondary antibodies.

7.3 Results

This section presents the summative results for the analysis of each of the selected detection methods. The results have been shown in previous chapters, as the detection methods were used in the cell line screening process, so the following sections present the results regarding the efficiency of each of the detection methods.

7.3.1 Analysis of SNAP-25 Standards

It is a requirement that the SNAP-25 standards made are pure enough to ensure detection of SNAP-25 specifically and robustly and the purity and specificity of the standards were analysed to ensure this. Figure 7.4 and 7.5 below show the Coomassie blue, and silver staining for the cleaved and whole SNAP-25 standards respectively at 1:10 and 1:100 concentrations (diluted in LDS sample buffer for Western Blot).

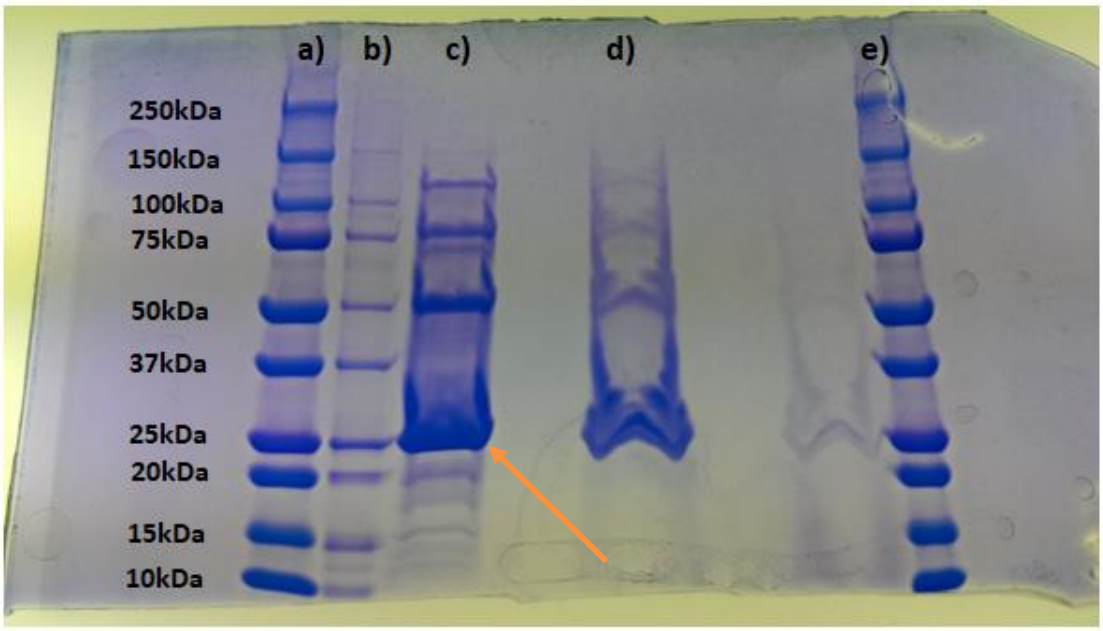


Figure 7.4: Coomassie Blue Staining of the Cleaved SNAP-25 Standard. *Cleaved SNAP-25 sits at 24kDa (labelled with an orange arrow).* ***(a)*** *Molecular marker (kDa)* ***(b)*** *overspill of marker* ***(c)*** *1:10 standard* ***(d)*** *1:100 standard* ***(e)*** *Molecular marker*

Migration issues with the Western blot tank caused oddly shaped bands, but overall, the staining indicates that there are many unspecific protein bands in the cleaved SNAP-25 standard, where only one band at 24kDa would be expected with a pure product (figure 7.4).

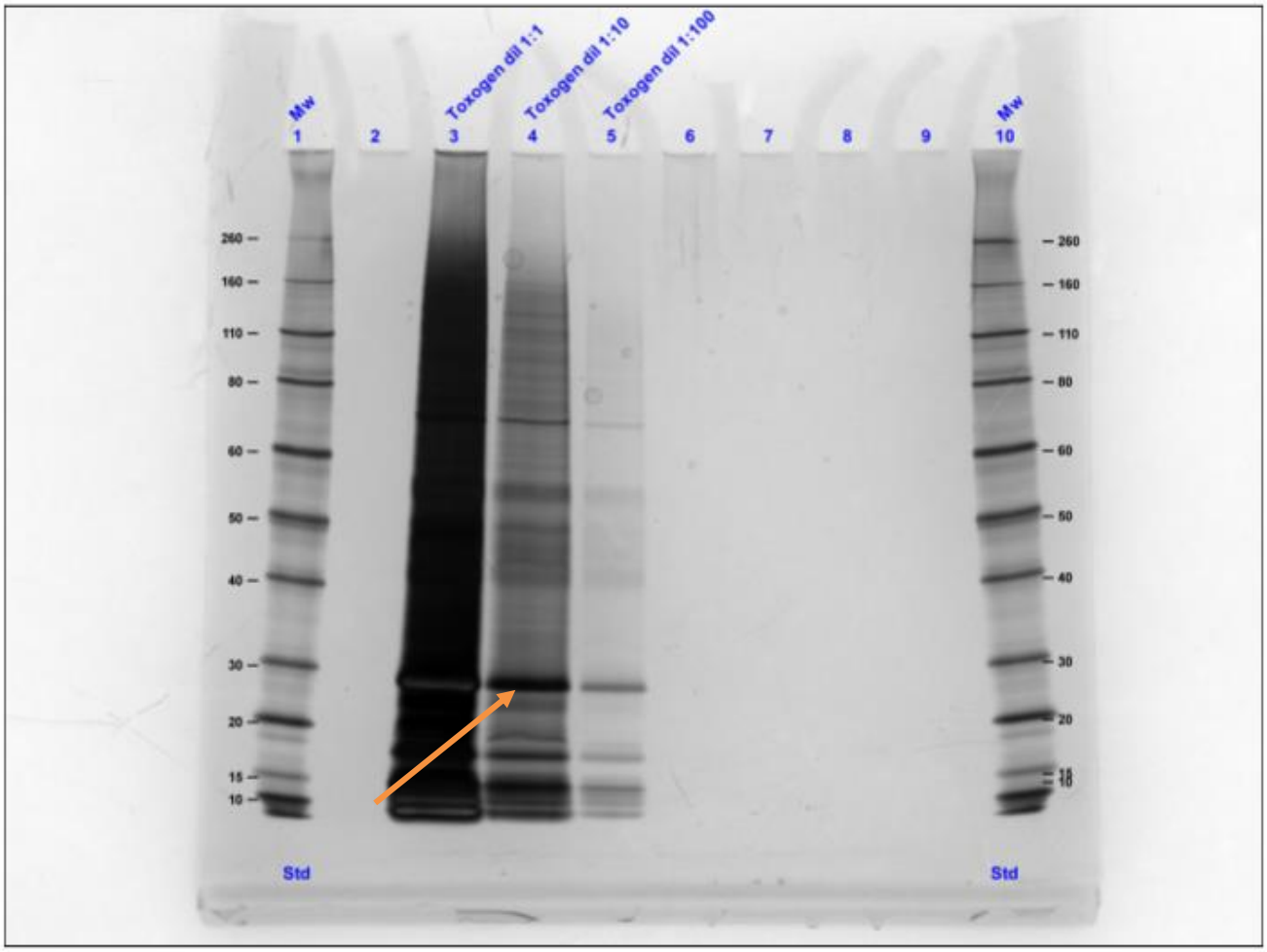


Figure 7.5: Whole SNAP-25 standard; silver staining demonstrating protein presence. *Image provided by project collaborator Uppsala University.* ***(1)*** *Molecular marker* ***(3)*** *Standard 1:1* ***(4)*** *Standard 1:10* ***(5)*** *Standard 1:100* ***(10)*** *Molecular marker. Arrow marks 25kDa highlighting detection of whole SNAP-25 band.*

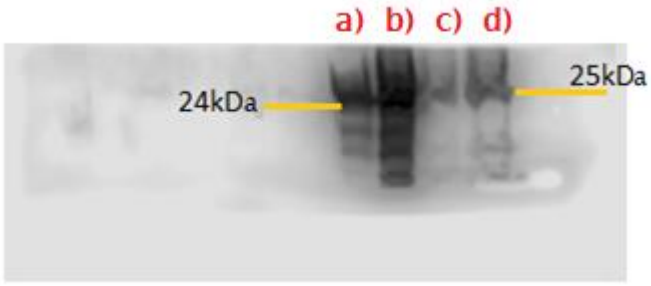


Figure 7.6: Western Blot analysis of whole and cleaved SNAP-25 standards *Anti-SNAP-25 polyclonal antibody detection (1:1000 concentration).* ***(a)*** *cleaved 1:100* ***(b)*** *cleaved 1:10* ***(c)*** *whole 1:100* ***(d)*** *whole 1:10.*

Figures 7.4, 7.5 and 7.6 above illustrate that both the whole and the cleaved SNAP-25 standards are not pure, with several bands detected at sizes different to the 25kDa or 24kDa expected bands. The specificity of the SNAP-25 antibody has been previously analysed; with no unspecific binding occurring for the cell sample analysis, so the antibody specificity has been confirmed and can be eliminated as a potential issue. It is thus concluded that the standards are not the purest form of cleaved or whole SNAP-25, as several bands can be detected on the Western blots of these standards, as shown by figure 7.6. A molecular marker was not used, in error, on this gel, but with specific standards you would expect to only see one clear band at 24kDa for the cleaved SNAP-25 standard and one band for the 25kDa for the whole SNAP-25.

7.3.2 Western Blot

The Western Blot detection method was used predominantly in the selection of a sensitive cell line, providing the ability to assess SNAP-25 expression, discussed in chapter 4, and SNAP-25 cleavage reflecting sensitivity to toxin in chapter 5. Blots are shown throughout the previous chapters, and with practise and optimisation, allowed for the presentation of clear blots, with the successful detection of SNAP-25 in both the uncleaved and cleaved forms. Results shown in chapter 5, section 5.3 only demonstrated the detection of the uncleaved SNAP-25 following toxin incubation, and the data was compared to the control 0U/mL

SNAP-25 expression which suggested cleavage. For more precise quantification it was necessary to optimise the methodology to ensure the detection of both cleaved (24kDa) and uncleaved (25kDa) SNAP-25.

As discussed previously, the duration of sample migration was extended to allow for further separation of the two bands and optimisation of the method was achieved. Figures 7.7 below shows Western Blot analysis of SNAP-25 before method optimisation for revelation of the cleaved bands and figure 7.8 shows examples of Western Blots with progressive improvement of cleaved SNAP-25 detection.

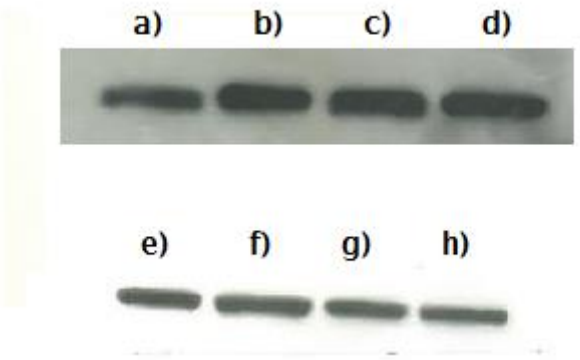


Figure 7.7: SNAP-25 Blots before Optimisation. *Detection of only the uncleaved SNAP-25 band. iCell neuron samples* ***(a)*** *30U/mL* ***(b)*** *0U/mL control* ***(c)*** *30U/mL* ***(d)*** *0U/mL control* ***(e)*** *Botox 30U/mL* ***(f)*** *Saline 0U/mL control* ***(g)*** *30U/mL* ***(h)*** *0U/mL control. SNAP-25 shown.*

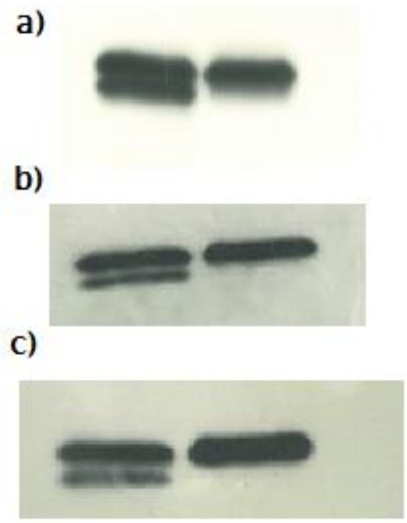


Figure 7.8: SNAP-25 Blots following Optimisation. *Detection of both the cleaved (lower band) and uncleaved (upper band) forms of SNAP-25 following toxin incubation 30U/mL (left lane) and 0U/control (right lane)* ***(a)*** *10 minutes extra migration* ***(b)*** *and* ***(c)*** *15 minutes extra migration.*

Figure 7.8 shows clear separation of the cleaved and uncleaved SNAP-25 bands in a sample treated with 30U/mL of toxin. The cleaved SNAP-25 shown as a 24kDa band can be distinguished from the remaining uncleaved protein at 25kDa. Figures 7.8b and 7.8c show improved separation than in 7.8a following a further increase in migration of the samples through the gel. This adaptation that improved the results was achieved by increasing migration to a total of 50 minutes, until the 15kDa molecular marker is at the base of the gel. The optimisation of the Western Blot methodology allowed for the detection and quantification of toxin activity, and slight changes to the protocol permitted selection of the most sensitive cell line; iCell neurons.

7.3.3 Mass Spectrometry

7.3.3.1 iCell Neuron Sample Results

A synthetic peptide for the 9-amino acid peptide cleaved from the C-terminus of SNAP-25 as a result of toxin activity was made and analysed alongside the iCell neuron samples. The identification of this 9-amino acid peptide would be a novel approach to detecting toxin activity, as it has yet to be shown what happens to this peptide following the toxin cleavage. To fully quantify the toxin activity using mass spectrometry it will be necessary to have a synthetic peptide control to the whole SNAP-25, cleaved 197 amino acid SNAP-25 peptide and detection of the 9-amino acid peptide would be an extra confirmation of activity.

Figure 7.9

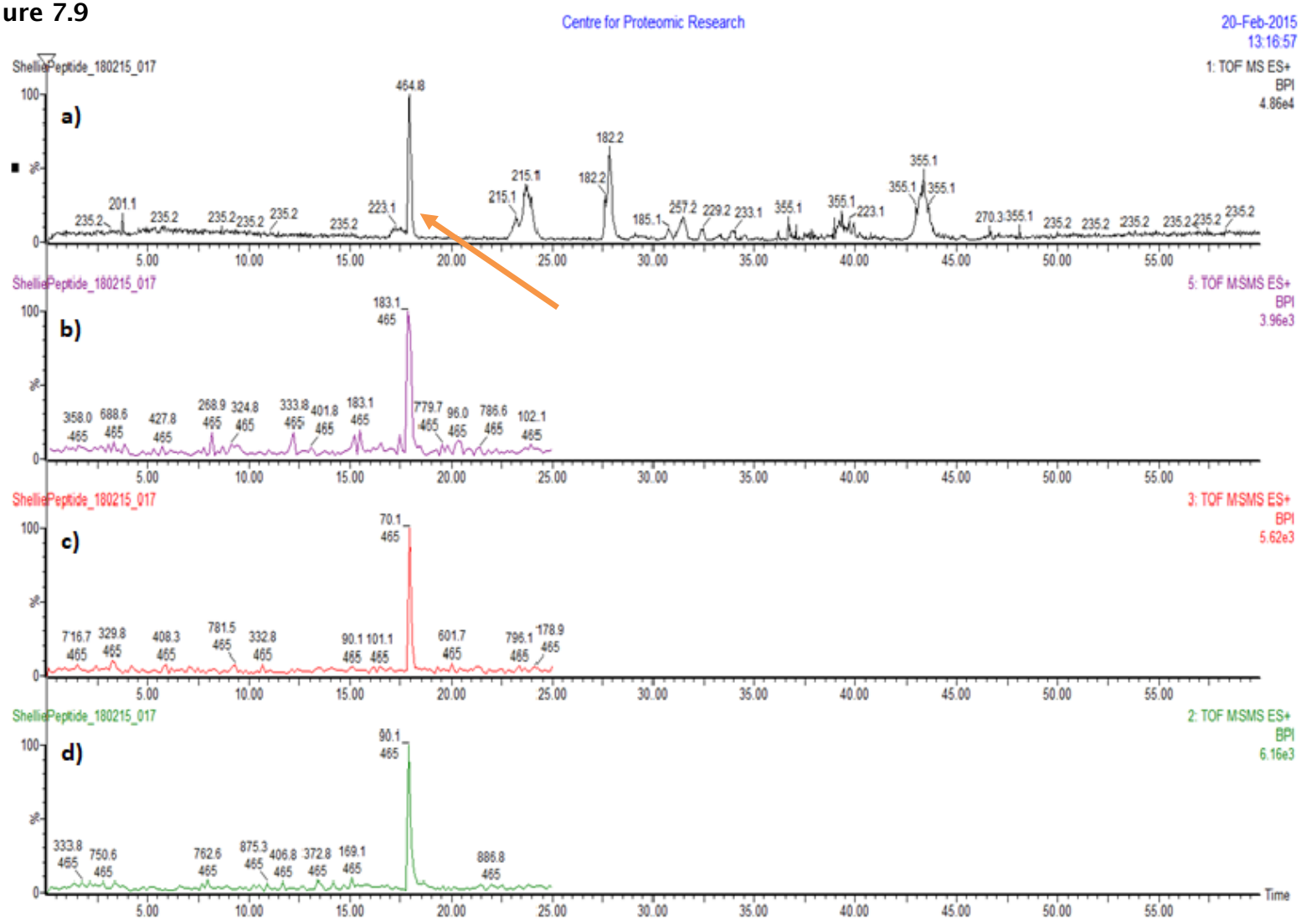

Centre for Proteomic Research
20-Feb-2015
13:16:57
ShelliePeptide_180215_017
1: TOF MS ES+
BPI
4.86e4
a)
464.8
182.2
215.1
355.1
ShellieP eptide_180215_017
5: TOF MSMS ES+
BPI
3.96e3
b)
183.1
465
ShellieP eptide_180215_017
3: TOF MSMS ES+
BPI
5.62e3
c)
70.1
465
ShellieP eptide_180215_017
2: TOF MSMS ES+
BPI
6.16e3
d)
90.1
465
Time

Figure 7.9: 9 amino acid reference peptide in iCell neuron cell lysate sample detected with mass spectrometer. *Base peak intensity and ion intensity mass to charge ratio (m/z). 9 amino acid peptide peak labelled with arrow* ***(a)*** *the base peak* ***(b) (c) (d)*** *three ion fractions corresponding to the base peak.*

Mass spectrum data is presented as a bar graph, with each bar representing an ion with a specific mass to charge (m/z) ratio and the y axis indicates the relative abundance of the ion. The most abundant ion is represented as 100 and is the base peak. Figure 7.9 demonstrates the mass spectrum data from analysis of the nine-amino acid reference peptide mixed with a complex cell sample (iCell neurons) and the figure shows that the reference peptide was successfully detected. It shows the base peak intensity; the base peak, and the three ion fractions detected corresponding to the base peak below. The analysis of iCell neuron samples, spiked with the reference peptide rendered inconclusive results; no other peptides were detected suggesting that SNAP-25 peptides, amongst other protein peptides expected in a complex cell sample, were undetectable. This could be a result of the current sample preparation method or the low cell density, contributing to the low abundance of these proteins in the samples. The samples used for mass spectrometry analysis were also analysed by Western Blot; SNAP-25 was detected so it is thus apparent that the cell density and subsequent protein concentration is not sufficient for the detection of SNAP-25 via mass spectrometry. We have negative results for the next two replicates of iCell neuron analysis with mass spectrometry; each experiment was unable to detect SNAP-25 peptide peaks in the samples or any peptides that correlate to the 9-amino acid reference peptide. Figures 7.10a and 7.10b below show the base peak and ion/ion fragments intensity on the mass spectrum (m/z charge) detected in the secondary analysis of a sample spiked with the reference peptide.

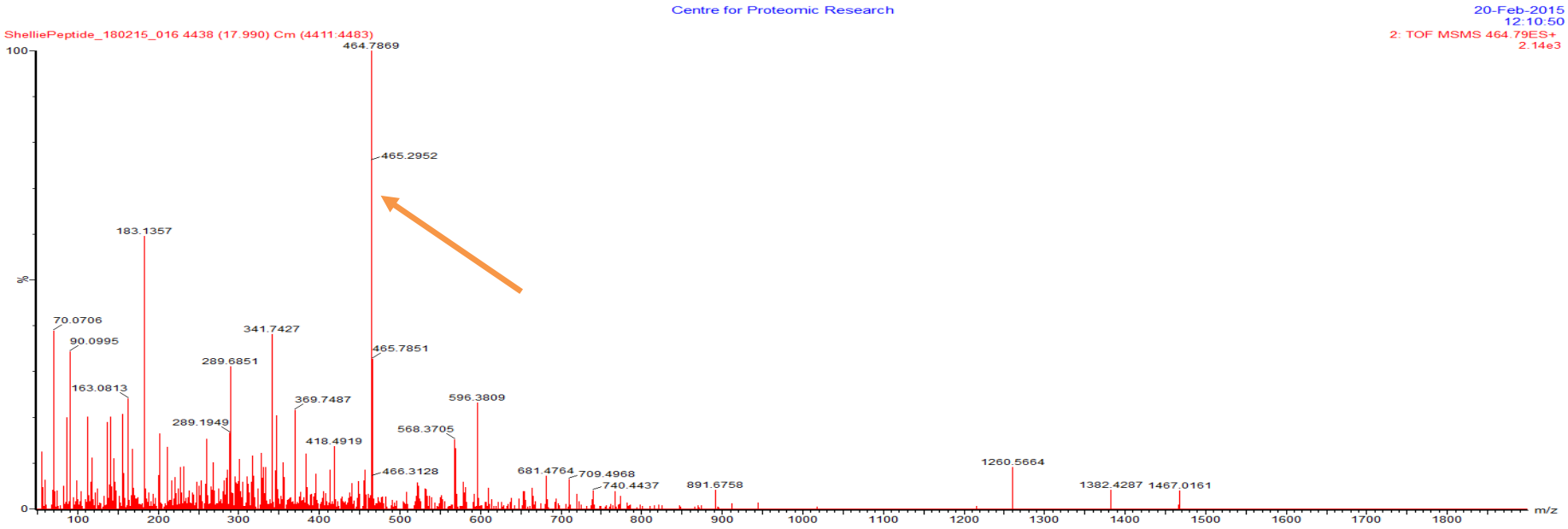


Figure 7.10: **(a)** *MS/MS Spectrum diagram. Ions detected with bars indicating mass/charge (m/Z) ratio. Y axis indicates relative abundance of ion. iCell neuron samples spiked with 9 amino acid reference peptide. Reference peptide peak labelled with an arrow.*

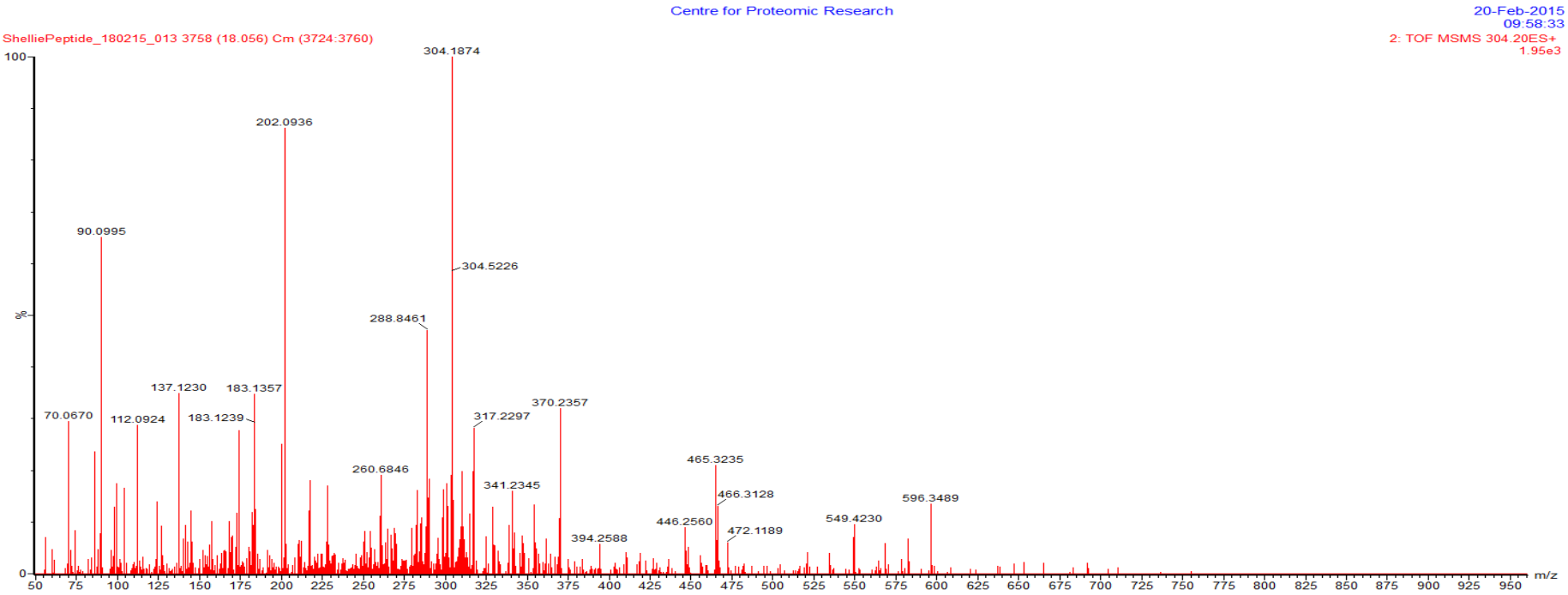


Figure 7.10: (b) *MS/MS Spectrum diagram. Ions detected with bars indicating mass/charge (m/Z) ratio. Y axis indicates relative abundance of ion. iCell neuron samples spiked with 9 amino acid reference peptide.*

Figure 7.10 illustrates the mass spectrometry analysis for an iCell neuron sample spiked with the reference peptide, in which only a few peptide ions were detected, shown with the mass spectrum diagrams. The obvious peak indicated with an arrow in figure 7.10a corresponds to the 9-amino acid reference peptide. It is expected that the results would be a lot more 'spiked' with copious peaks detected for different peptides in the complex analyte, suggesting an issue in the sample preparation and peptide filtration step. Following this, it was decided to increase the protein concentration of the samples by increasing the cell density; results are shown below in figures 7.11 and 7.12. We used neuroblastoma cell lines for this optimisation, as the iCells are too expensive to trial on repeated high cell density sample analysis to optimise the mass spectrometry approach.

7.3.3.2 Neuroblastoma High Cell Density Sample Results

Two samples from each of the neuroblastoma cell lines; BE(2)-C, SH-SY5Y and IMR-32, at high cell density, were prepared. Although the IMR-32 cell line had been discarded for maintenance issues, this sample yielded one of the highest total protein concentrations before analysis with mass spectrometry and was therefore used to optimise the methodology. The cell number and protein concentrations for each sample are shown in table 7.7.

Sample	Protein Concentration µg/µl	Number of Cells/sample
SH-SY5Y 4% SDS	1.941	$1x10^6$
BE(2)-C 4% SDS	**8.804**	$5x10^6$
IMR-32 4% SDS	**11.824**	$3x10^6$
SH-SY5Y 0.1% SDS	0.755	$1x10^6$
BE(2)-C 0.1% SDS	6.144	$5x10^6$
IMR-32 0.1% SDS	1.519	$3x10^6$

Table 7.7: Protein concentrations of prepared samples. *Two highest concentrations selected for analysis with mass spectrometry highlighted in red.*

Firstly, we performed the peptide analysis of the highest protein concentration sample; the IMR-32 4% SDS cell sample. Following OFFGEL fractionation, analysis of the subsequent 12 fractions generated was carried out on the Orbitrap fusion model mass spectrometer. The analysis showed only 24% peptide coverage for whole SNAP-25, and the peptide containing the amino acid sequence incorporating the 197-amino acid cleavage site was not detected, shown in figure 7.11.

To try and improve the detection coverage and increase the chances of detecting the peptide of interest that includes the toxin cleavage site, the BE(2)-C sample,

with a higher cell density than the IMR-32 cell sample previously analysed, was processed (figure 7.12).

Figure 7.12 data was generated from the analysis of the high-density BE(2)-C cell line sample; following OFFGEL fractionation, and subsequent analysis of the 12 fractions generated, on the Orbitrap fusion model mass spectrometer; the same conditions as the IMR-32 sample. The analysis shows an improved 54% peptide coverage for whole SNAP-25, compared to the 24% coverage for the IMR-32 $3x10^6$ cell density sample, but the peptide containing the cleavage site was still not detected. The peptide that incorporates the cleavage site of Botulinum toxin type-A is underlined in red on the amino acid sequence on figure 7.12. This peptide was not detected in either the IMR-32 or the BE(2)-C samples; both of which yielded the highest total protein concentrations. This suggests that the other cell line samples, with lower protein concentrations, would not provide improved results if analysed.

The results of the peptide analysis of the IMR-32 and the BE(2)-C cell line samples are shown in figures 7.11 and 7.12 below.

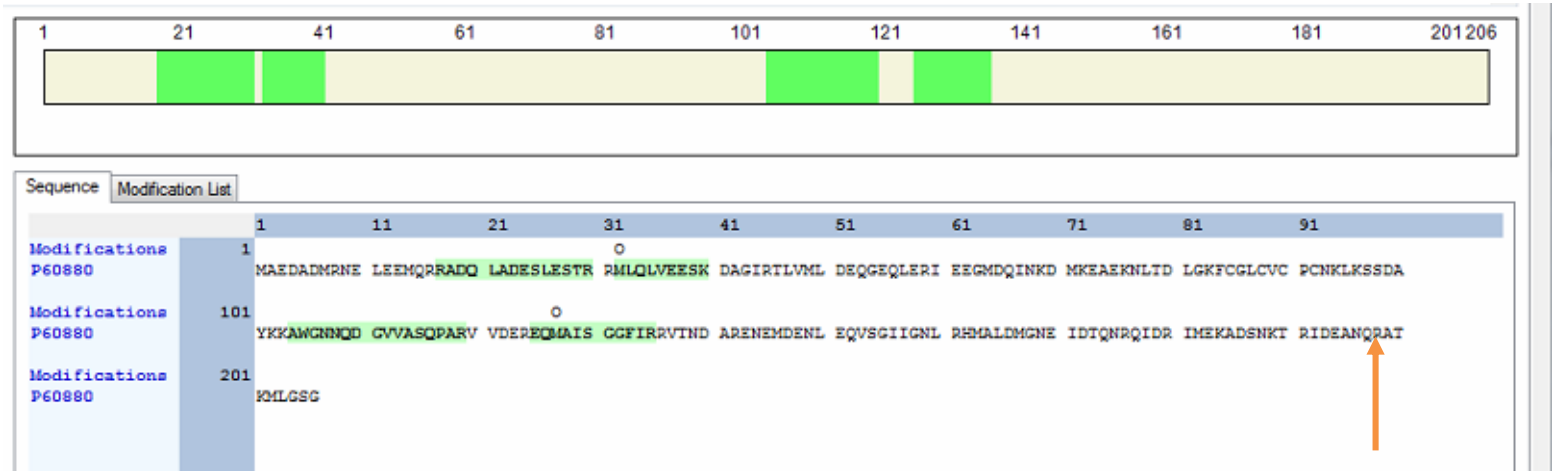


Figure 7.11: SNAP-25 peptides detected in IMR-32 high cell density (3 million cells) sample in TEAB 4% SDS. *The detected peptides are highlighted in green- 24% peptide coverage. The cleavage site of BoNT-A is shown with an arrow, and the peptide encompassing this site was not detected.*

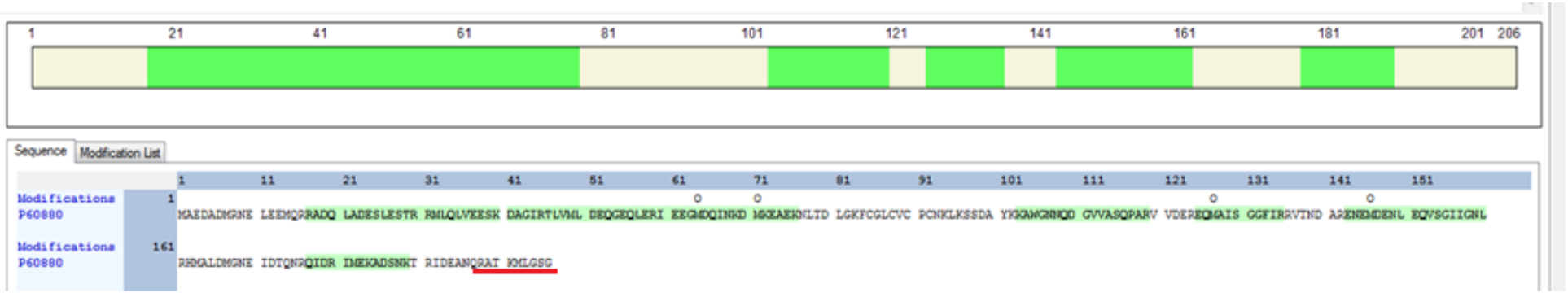


Figure 7.12: Peptides detected in BE(2)-C high cell density (5 million cells) sample in TEAB 4% SDS. *The detected peptides for SNAP-25 are highlighted in green. There is 54% peptide coverage of SNAP-25. Peptide that is cleaved as a result of toxin type-A activity is underlined in red. The toxin cleaves at the C-terminus of SNAP-25, between Q and R, cleaving 9 amino acids.*

7.3.4 Microchip Electrophoresis

7.3.4.1 Confirmation of Primary Antibody Labelling

The anti-SNAP-25 polyclonal antibody (Sigma) was labelled using a Lightening Link 488 Kit, as per the manufacturer instructions. To analyse the success of the labelling, the antibody was incubated on a Western blot membrane. The nitrocellulose membrane was incubated with the labelled primary antibody overnight, and analysed with a fluorescent microscope to detect the protein bands. The results of this Western Blot concluded that the white membrane, to which the protein and antibody is bound, produces too much interfering background for detection under a fluorescent microscope; which did not allow for fluorescent detection of SNAP-25 bands.

As the success of the labelling of the primary antibody cannot be determined through Western Blot analysis is was agreed to proceed to the secondary amplification step straight onto the microchip platform. Samples were incubated in both the primary, unlabelled antibody and a fluorescently tagged secondary antibody, as discussed below.

7.3.4.2 Signal Amplification with Primary and Secondary Antibodies Experiments

Western Blot has a two-stage signal amplification protocol, with a labelled secondary antibody added for increased signal. Using only a primary labelled antibody is a reduction in amplification and with the proven small abundance of SNAP-25 in the samples, an amplification step may be necessary. Alexa 488 fluorescent secondary antibody was added to the primary antibody and sample mixture to encourage detection of SNAP-25 in a format similar to the successful Western Blot.

When analysed on the microchip, a fluorescent band was detected for each sample of whole or cleaved SNAP-25 standards combined with both primary and secondary antibodies (see figures 7.13 and 7.14); but the method, despite optimisation of gel and applied voltage, did not allow for sufficient separation

of the antibody and identifiable SNAP-25. The detected bands each correspond to a combination of whole or cleaved SNAP-25 and the primary and secondary antibodies bound together, figures 7.13 and 7.14 below show the detected fluorescent bands for each of the experimental conditions described in the method section. Each experiment was carried out three times, each with similar results, showing that the separation of cleaved and whole SNAP-25 standards with this approach was not successful. Separation, in relation to these experiments, refers to both the capability of defining separation of each of the standards from the residual antibodies and also defining separation of cleaved and whole SNAP-25 when combined together. The latter is comparable to the final assay approach; as distinction between the two forms in the same sample is a necessity for successful assessment of toxin activity.

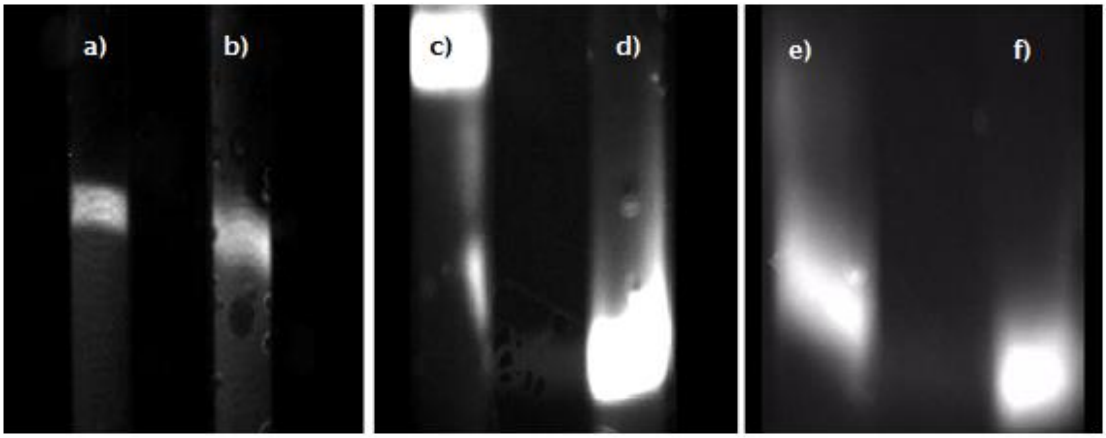


Figure 7.13: Fluorescent detection of SNAP-25 using microchip separation. *All images show 7nL of sample diluted 1:1 in LDS sample buffer, incubated in primary and secondary antibody analysed in a microchip with 2.5% PEO gel. 170V/cm voltage applied.* ***(a)*** *cleaved SNAP-25 standard* ***(b)*** *uncleaved SNAP-25 standard* ***(c)*** *cleaved SNAP-25 standard 3cm microscope view* ***(d)*** *uncleaved SNAP-25 standard 3cm microscope view* ***(e)*** *cleaved SNAP-25 standard 6cm microscope view* ***(f)*** *uncleaved SNAP-25 standard 6cm microscope view.*

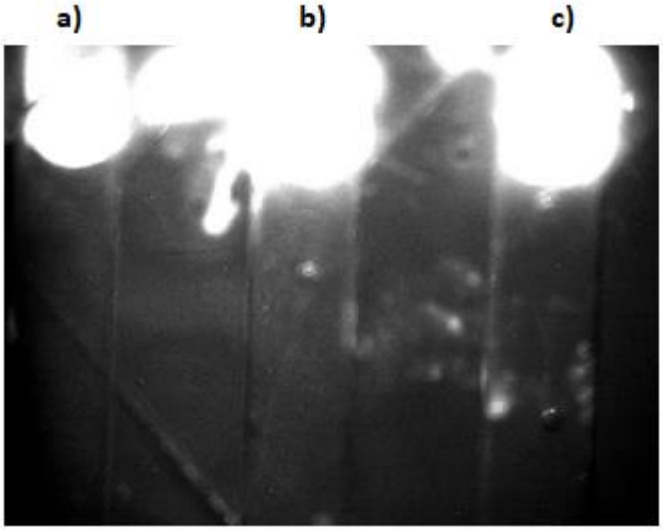


Figure 7.14: Detection of a fluorescent signal corresponding to a complex formed by SNAP-25 standards and primary and secondary fluorescent antibodies. *100nL of each sample loaded into channels* ***(a)*** *Whole SNAP-25 standard* ***(b)*** *Cleaved SNAP-25 standard* ***(c)*** *Whole and cleaved SNAP-25 standards combined.*

Figure 7.14 above shows three replicates of the experimental design of 7nL of protein standard sample, mixed with primary and secondary antibodies, loaded into the gel channels. The detection chip should separate the samples into different fluorescent bands dependant on molecular weight; unbound primary and secondary antibodies and SNAP-25 (cleaved and uncleaved) tagged with the primary and secondary antibodies. As these images show, the fluorescent band does not separate during migration, preventing the detection of tagged SNAP-25 separately to unbound antibody.

This was then increased to loading 100nL of samples to wider channels in the microchip with a reduced voltage to try and encourage separation of the SNAP-25 protein and the antibodies to allow for quantification of cleaved and uncleaved SNAP-25, shown in figure 7.14. This image shows clear detection of a fluorescent band in each channel, but distinction between separate bands in the same sample is still not achieved.

7.4 Discussion

As defined in the introduction to this chapter, there are standards and criteria that should be applied to the detection methods in the process of selecting the most suitable candidate to ensure that the assay as a whole would be suitable for regulatory submission. To enable the assessment of these criteria against each of the selected detection methods, stop or no/go points were defined to ensure each method was able to meet the set criteria for use in a cell-based assay for the potency testing of botulinum drug product.

SNAP-25 standards: A reference standard is an important control for use in a drug product release assay for use with the selected detection method; to validate the results, identify detection of the correct protein (SNAP-25) and provide a control to ensure the technique is working. The standards for whole and cleaved SNAP-25 produced for this project have demonstrated poor purity and analysis of a commercially available whole SNAP-25 peptide (Abcam), by a collaborating research group, provided results that showed similar impurity (results not shown). The results in section 7.3.1 show that the both the whole and the cleaved SNAP-25 protein standards are highly impure, when analysed with coomassie or silver staining and in Western Blot. The antibodies used in this project have always provided specific detection and clear blots that have minimal background detection, and the protein bands for SNAP-25 are usually well defined. The Western Blot analysis for the whole and cleaved SNAP-25 standards provided smeared bands, with various bands detected by the antibodies at different molecular weights. Although the whole SNAP-25 standard was slightly improved in terms of specificity for the Western Blot, there were still multiple bands detected in the silver staining of the product.

This could potentially highlight the difficulty in producing a control protein for SNAP-25, and the current standards could not be used in a GMP compliant assay. For screening of detection methods such as Western Blot and the Microchip electrophoresis methods, the standards are acceptable as separation of the proteins based on molecular weight in a gel can allow for quantification of only the 25kDa or 24kDa protein. To use these standards with mass spectrometry however could prove challenging, given the clear impurities in the cleaved and uncleaved standards and these reference proteins will have to be purified or new ones synthesised.

Western Blot: This detection method proved to be a prominent tool in the cell screening process, providing indicative results that allowed the selection of the iCell neurons as the most sensitive cells based on the detection of SNAP-25 cleavage. The first criteria for the selection of a suitable cell line was that the cell line expressed SNAP-25 and the Western Blot method successfully detected whole SNAP-25 in cell line samples, and allowed for the reliable elimination of unsuitable candidates. Chapter 5 indicates the first issues with the detection method, demonstrating that the detection and distinction between cleaved and uncleaved SNAP-25 was problematic with the original methodology. Optimisation and alterations to the approach of analysis, with reference to change in gel concentration and gradient, cooling of the buffers and increasing the gel migration time to ensure separation of cleaved and uncleaved SNAP-25. This allowed for more detailed experimentation into toxin sensitivity of each cell line and the Western Blot was an ideal detection method for these requirements. However, when looking at the requirements of a detection method, and an assay as a whole, accuracy, robustness and repeatability cannot be applied to the Western Blot method. The quantification of the results, using ImageJ is somewhat subjective as the bands are manually selected and this could be perceived as a variable. The protocol, although not specifically complex, is subject to variation and possible error, with particular reference to the transfer of the proteins to the membrane. Many steps in the protocol could be exposed to variation and the process of optimisation of the methodology highlights several concerns for using Western Blot as the selected detection method. Although its apparent accuracy should not be questioned, as the method allows for the normalisation of protein expression results with the use of a protein control, Beta-Actin in this project, the robustness of the method and the potential for variance in results and reliability suggested that the Western Blot should not be considered for use in the final cell-based assay.

Table 7.8 below summarises the application of the defined criteria in selecting a suitable detection method to the Western Blot methodology.

Criteria		Western Blot Results/Observations
Accuracy		Reliable controls in place to ensure accurate detection of SNAP-25. Specific antibodies that reliably detect protein of interest.
Precision	Repeatability	Method was not always replicable using the same samples; open to error, variable detection using film.
	Reproducibility	Method was carried out in a different laboratory- area in which the experiment is conducted does not have an effect on the method- variables are within the protocol.
Robustness		Method is not robust, and required extensive optimisation to provide results; which are not generated consistently with each analysis in some cases.
Detection Limit		The method suggested a detection limit, showing apparent cleavage at 0.1U/mL, but the cleaved SNAP-25 could not be detected reliably.
Linearity		Considering the above criteria and the unsuitability of the method, these criteria were not assessed.
Range		

Table 7.8: Defined Criteria for Selection of a Suitable Detection Method Applied to Western Blot.

Mass Spectrometry: The study by Brinkmalm, et al, 2014, demonstrated that SNAP-25B specific peptides were far more abundant than those of SNAP-25A and other studies have confirmed that SNAP-25B is the predominant isoform in the adult brain (Yamamori, Itakura et al. 2011, Brinkmalm, Brinkmalm et al. 2014). Interestingly, the results from this paper (Brinkmalm, Brinkmalm et al. 2014) showed successful detection of SNAP-25B in each of their samples, only with coverage of the amino acids 2-198, similarly to our results for mass spectrometry so far. SNAP-25 is tightly formed with SNARE complex proteins and strongly associated with the cell membrane (Brinkmalm, Brinkmalm et al. 2014). It is difficult to analyse membrane associated peptides via mass spectrometry, as this technique is incompatible with most detergents used to solubilise membrane bound proteins. These results suggest that detecting the peptide of interest in terms of toxin activity may prove difficult, if not impossible. The analysis of the iCell neurons samples, spiked with a reference peptide for the 9 amino acid cleaved fraction of SNAP-25, did not provide any conclusive results. The reference peptide could be detected, but this is not found in the cell sample. It is still unknown what happens to the cleaved 9 amino acids of SNAP-25 as a result of cell exposure to toxin, but it is confirmed by our results that it is not detectable in a sample following sample preparation for mass spectrometry analysis.

The abundance of protein is an issue in our project, with the selected cell line being expensive and difficult to provide high density samples, so detection of SNAP-25 could be challenging. The analysis of the high density neuroblastoma cell line samples indicated that detection of SNAP-25 peptides was possible, with the best results showing 54% of peptide coverage, but the peptide of interest, that incorporates the toxin binding site, was not detectable in each of the experiments. This renders the methodology unusable, as this particular area of the peptide structure is the area of interest. Without distinction between cleaved or uncleaved SNAP-25, or the ability to assess whether the toxin has bound to and entered the cells resulting in the cleavage of 9 amino acids from the C-terminus, the method cannot provide the appropriate data for a cell-based assay detection method. The regulatory submission criteria were applied to the mass spectrometry method and summarised in table 7.9 below.

Criteria		Mass Spectrometry Results/Observations
Accuracy		Reliable controls in place to ensure accurate detection of SNAP-25. Renowned method for specificity and accuracy.
Precision	Repeatability	Method was not always replicable using the same samples; iCells no peptides detected with original approach.
	Reproducibility	It was not possible to reproduce the data in a different laboratory setting and only one mass spectrometer was available for us- this has not been assessed.
Robustness		Method is robust, but required extensive optimisation to provide results. The method is not suitable for the project requirements.
Detection Limit		Considering the above criteria and the unsuitability of the method, these criteria were not assessed.
Linearity		
Range		

Table 7.9: Defined Criteria for Selection of a Suitable Detection Method Applied to Mass Spectrometry.

Microchip Electrophoresis (MCE): The microchip detects proteins in droplets with fluorescence under a microscope. The primary antibody used to detect uncleaved and cleaved SNAP-25 for the Western Blot analysis is one of the few commercially available and currently there are no fluorescent antibodies with affinity to both forms of SNAP-25 required for the quantification of toxin activity. Labelling this optimised antibody with a fluorescent tag makes it adaptable for use with this detection technique. Figures 7.13 and 7.14 in the results section showed three replicate experiments, each detecting a singular fluorescent band in each of the sample channels. The distinction between cleaved and uncleaved in the mixed standards sample was not possible; a compulsory element to the final assay design. The mixed cleaved and uncleaved SNAP-25 standards sample was the clearest indication that this methodology may not be suitable for our project needs, given that the samples analysed for routine testing will require the distinction between cleaved and uncleaved in the same sample for analysis of toxin activity. The methods inability to separate these forms of SNAP-25 indicates that, despite optimisation of migration voltage, gel concentration and sample volume, the detection of toxin activity will not be possible using MCE.

When comparing the MCE method to the criteria defined in the introduction to this chapter (shown in table 7.10) it is clear that the method would not be suitable for the testing of a toxic drug product. The method cannot be deemed accurate, as the fluorescent band detected was a combination of both antibody and SNAP-25 and there was no way in which to quantify the amount of each based on the migration results. Although the method is clearly repeatable, given the results of each experiment were the same, this cannot be confirmed as the data provided did not provide any conclusive observations in relation to amount of SNAP-25 detected. Table 7.10 below provides a summary of the criteria against the Microchip Electrophoresis detection method.

Criteria		Microchip Electrophoresis Results/Observations
Accuracy		Our results did not suggest method accuracy, with no specific detection of SNAP-25.
Precision	Repeatability	Method provided the same results each time; but these were not suitable for the assay (just detected one fluorescent band).
	Reproducibility	It was not possible to reproduce the data in a different laboratory setting and only one microchip was available- this has not been assessed.
Robustness		Method required extensive optimisation to provide results. The method is not suitable for the project requirements.
Detection Limit		Considering the above criteria and the unsuitability of the method, these criteria were not assessed.
Linearity		
Range		

Table 7.10: Defined Criteria for Selection of a Suitable Detection Method Applied to Microchip Electrophoresis.

To summarise, each of the methods tested failed to meet the criteria necessary for the regulatory submission of a cell-based assay detection method. The specifications and requirements shown in table 7.11 provide an indication as to when each detection method failed to meet criteria. MCE failed to show detection of whole SNAP-25, and experiments were also indicative that separation of cleaved and uncleaved SNAP-25 as a result of toxin activity would not be possible. The analysis was ceased at criteria 2. Mass Spectrometry also followed a similar suit, whole SNAP-25 was detected, but the peptide of interest was not found after multiple experiments, this analysis ceased before investigation of distinction between cleaved and uncleaved SNAP-25. The Western Blot was the most successful, providing detection of whole SNAP-25, distinction of cleaved and uncleaved and the provision of reliable and repeatable results with the selected cell line. However, given the specific accuracy requirements of the method, it was suggested that the method would not be GMP compliant for this particular testing. The many variables of the method, and a quantification method that can be inconstant, the Western Blot would not be suitable for the potency testing of Botulinum Toxin Type-A.

Criteria	Specifications/Requirements	Stop/no-go points- apply to all methods
1	Detection of Whole SNAP-25 ✓ ✓ x	• Detection of whole SNAP-25 not possible **MCE ceased** • Distinction between cleaved and uncleaved SNAP-25 not achieved **Mass Spec ceased** • GMP compliant method/quantification not feasible. **Western Blot ceased**
2	Distinction between cleaved and uncleaved SNAP-25 ✓ x x	
3	Repeatable and reliable results with the selected cell line ✓ x x	
4	Reliable quantification and determination of potency x x x	

Table 7.11: Criteria for the Selection of a Detection Method. *Each method shown in colour Western Blot, Mass Spectrometry, Microchip Electrophoresis.*

7.5 Conclusions

Based on the observations of each of the detection methods screened, when applying the criteria and principles set out in the approach to selecting an appropriate method, each has its benefits and disadvantages. Western Blot, MCE and Mass Spectrometry were all selected based on excelling features that suggested suitability for a cell-based assay for the potency testing of Botulinum toxin type-A. Western Blot proved the most useful, in relation to selection of the most sensitive cell line, but the method would not be able to be used in a routine GMP-compliant drug product release assay and therefore it was discarded as a candidate. Mass spectrometry successfully demonstrated detection of SNAP-25, but the protein concentrations required for the detection of this low abundance proteins would not coincide with the selected cell line. Also, the most important peptide to assess toxin activity was not ever detected, despite an increased protein concentration with the high-density cell samples. This provided cause for elimination of mass spectrometry for potential use in the final cell-based assay. Our final detection method was also, unfortunately, unsuccessful. The high sample throughput and small volumes of samples needed for analysis were intriguing for use in the complete assay, but the sensitivity of the method, despite optimisation, was not great enough for our uses. Separation of the cleaved and uncleaved SNAP-25 as a result of toxin exposure, was not possible in several analyses, deeming the method also unsuitable.

The research into these different detection technologies has provided opportunity for industry to possibly expand their capabilities in the future for different projects but each method was deemed unsuitable for the testing of Botulinum toxin type-A activity. Given the high potency, and potential dangers of this drug product, a detection method must be reliable, repeatable and robust enough to ensure drug potency accuracy and subsequent patient safety; each of the trialled methods would not provide this.

We have a new method in the pipeline, which is awaiting sponsor funding and approval, which will hopefully provide the optimum detection method for this assay design and this is discussed in chapter 8.

Chapter 8: Moving Forward

8.1 Future Assay Development

As the need for the replacement of animal testing methods for the pharmaceutical industry is ever pressing, the continuation of this project is of upmost importance. The extensive work has thus exceeded the project timelines for both the Knowledge Transfer Partnership and completion of this thesis, but continued client sponsorship, and industry support will allow for the continuation of research into an *in vitro* alternative for the potency testing of Botulinum toxin type-A at Wickham Laboratories Ltd. This project has allowed for the successful selection of an optimal cell line for use in the assay format, and it was unfortunate that the selected detection methods, after careful analysis, would not be suitable for the project needs. The selection of this detection method, as discussed in Chapter 7, is perhaps more challenging than originally anticipated, given the difficult nature and detection of SNAP-25 and subsequent toxin activity. This is the remaining step in the completion of a successful assay, and another method has been proposed as a potential candidate.

Moving forward, the focus on the continued project will be to optimise a sensitive and suitable detection method that can be used for the GMP potency cell-based assay. Toxin activity was detected at levels as low as 0.1U/mL using the Western Blot methodology but, as previously discussed, Western Blot is not a technique that could be validated according to FDA requirements for the assay. The following section introduces a new method for screening, and further funding from both Wickham Laboratories and a sponsoring client will allow research into these avenues.

8.2 Proposed Detection Methods and Ongoing Research

8.2.1 ELISA

The sandwich ELISA method, detecting both cleaved and uncleaved SNAP-25, has been used in a submitted cell-based assay from a competing company (Fernandez-Salas, Wang et al. 2012). It has been shown to work yet is not readily available to all companies who test Botulinum toxins. This methodology will require the generation of unique and non-patent encroaching antibodies, specific for cleaved and whole SNAP-25, which will be an expensive and extensive process. The company who have submitted the cell-based alternative to the mouse potency assay have patented the methodology that incorporates '*antibodies that recognise the cleavage site of Botulinum toxin type-A on SNAP-25*', making the method problematic to employ as an independent company.

The ELISA method, however, has been put forward as a method of interest for this project based on the confirmation of use in another similar assay, and the ability to QC-validate the method and ensure GMP compliance is appealing. Extensive discussions with the client and sponsoring company have led to the agreement that the method is utilisable and would be suitable for our requirements; and we have made some suggestions that would enable avoidance of patent infringing methodologies, discussed in later sections.

8.2.2 Affimers to Replace Antibodies

Antibodies are tools most commonly used for studying protein expression and have, for many years, been accurate and beneficial but there are growing concerns about the difficulty surrounding sourcing and validating antibodies (Tiede, et al, 2017). The process of making antibodies is time-consuming and costly, so alternatives that could offer a cost-effective and rapid renewability have been studied in recent years. These 'renewable binding reagents' are thus preferred as they are recombinantly produced from a known sequence and affimers fall into this category.

Affimers have been used in research in a number of assays including immune-like affinity assays and have shown ability to be expressed in mammalian cells to manipulate cell-signalling.

For this project, we have been collaborating with a company who specialise in affimer design and have designed a protocol for the production of affimers specific to both the whole and cleaved forms of SNAP-25 as a direct replacement for antibodies currently used. This is intended to avoid all possible encroaches of the patented method and will enable the use of an ELISA method without infringing on patent restrictions.

The follow-up project therefore, has two aims; to identify affimer reagents that specifically bind to the full-length form of human SNAP-25 and the secondary aim is to identify affimer reagents that specifically bind to the truncated form or cleaved form of human SNAP-25.

The full methodology with the approach to the screening process and selection of appropriate affimers and all other intended work is confidential and property of Wickham Laboratories Ltd so is therefore not discussed in this thesis.

Appendices

Appendix A	KTP Project Timeline
Appendix B	Haemocytometer
Appendix C	Molecular Ladder – Western Blot
Appendix D	Western Blot Transfer Protocol
Appendix E	Antibodies Used in Project
Appendix F	Western Blot Antibody Incubation Protocol
Appendix G	ECL Revelation Protocol
Appendix H	Quantification with Image J

Appendix A : KTP Project Timeline

Project Stage 1	Project Stage 2		Project Stage 3	Project Stage 4		Project Stage 5			Project Stage 6		
Oct-Dec 14	Dec-Mar 15	Mar-June 15	June-Sep 15	Sep-Dec 15	Dec-Mar 16	Mar-June 16	June-Sep 16	Sep-Dec 16	Dec-Mar 17	Mar-June 17	June-Sep 17
Induction & Risk Assessment	Select Cell Lines	Analysis with WB	Detection Method Lit Review	Initial trialling of methods		Selection of Equipment			Final Qualification		Final Report
KTP Module 1	Initial Testing		Selection	Select most sensitive option		Set up In Vitro Lab			Validation Tests		
Market Assessment	Testing several cell lines			Refine method		Training of Staff			Regulatory Submission		
Design Parameters	Analysis of Sensitivity					Initial Report					
Facility Report	KTP Module 2					Optimisation of assay					

Appendix B : Haemocytometer

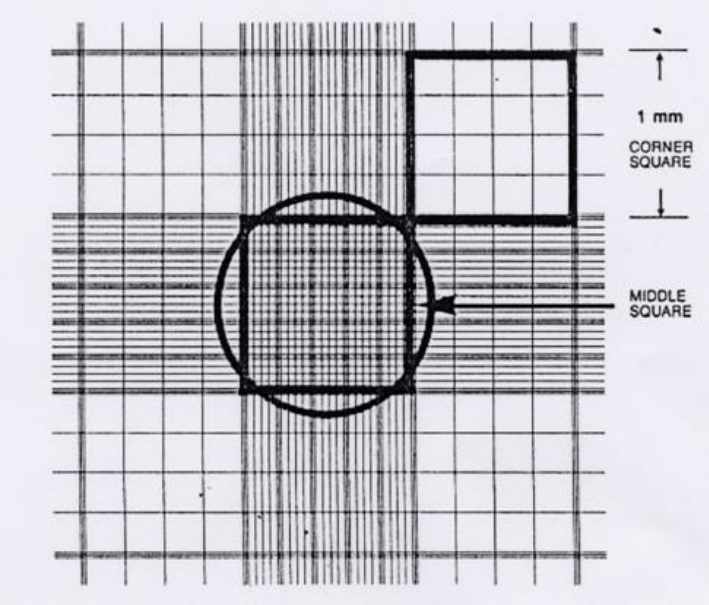


Figure Appendix B.1. *Appearance of the haemocytometer grid visualised under the microscope. Images taken from Public Health England* https://www.phe-culturecollections.org.uk/technical/ccp/cellcounting.aspx

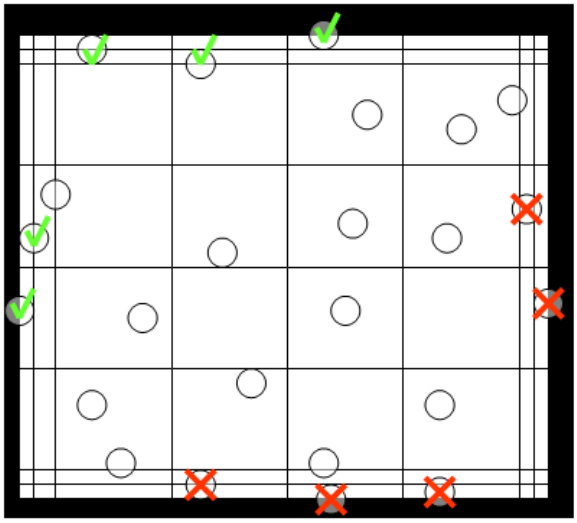
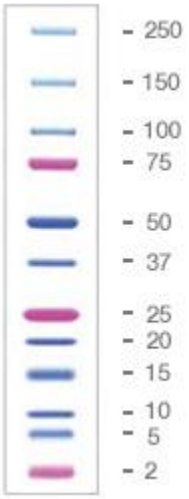


Figure Appendix B.2: *Counting system to ensure accuracy and consistency. Count the cells within the large square and those crossing the edge on two out of the four sides.*

To calculate cell concentration per mL: (volume of 1 square = 0.0001mL)

Number of live cells / number of corner squares counted x trypan blue dilution factor (2) x 10,000.

Appendix C Molecular Ladder- Western Blot



BioRad Precision Dual Colour Molecular Weight Marker

Supplier: BioRad, 500µL

Product Code: #1610374

Appendix D Western Blot Transfer Protocol

Transfer to Membrane

- Prepare Blotting Buffer
  3.03g TRIS, 14.4g Glycine, 200mL Methanol, 800mL distilled water.
- Remove the gel from the plastic casing, after cutting the corner for identification, remove the wells and the blue LDS buffer line at the base of the gel and submerge in the blotting buffer for 15 minutes.
- Submerge two sponges (per gel) and two filter papers (per gel) in blotting buffer and pre-wet a piece of membrane.
- Load the red transfer cassette, the order is crucial for a successful transfer;

Sponge foam pad
Filter paper
Membrane
Gel
Filter paper
Sponge foam pad
Black base of cassette inserts

- Roll out any air bubbles and load the cassette into the tank.
- Add the freezing unit and a magnetic stirrer to the tank.
- Fill the chamber with blotting buffer and attach the power pack 100V for **one hour**. This timing is crucial to prevent errors.

Appendix E Antibodies Used in Project

Antibody	Provider	Concentration
Rabbit anti-SNAP-25 polyclonal antibody	Synaptic Systems #111 002	1:1000
Mouse anti-SNAP-25 monoclonal antibody	Synaptic Systems #111 111	1:1000
Mouse anti-SNAP-25 monoclonal antibody	Synaptic Systems #111 011	1:1000
Rabbit anti-SNAP-25 polyclonal antibody	Sigma, #S9684	1:1000
Rabbit Beta-Actin (housekeeping protein) antibody	Synaptic Systems #251 003	1:20000
Beta-III-Tubulin (mouse)	Covance mms-435P	1:500
ChAT antibody (goat)	Merck Millipore AB144P	1:50
MNR2 (mouse)	Developmental studies Hybridoma bank 81.5c10	1:1, 1:5
Rabbit anti-mouse IgG (H+L) Alexa Fluor 488	ThermoFisher #A27022	1:200
Rabbit anti-goat IgG (H+L) Alexa Fluor 568	ThermoFisher #A27011	1:200

Appendix F Western Blot Antibody Incubation Protocol

Primary Antibody Incubation

- After one hour, turn off the power pack, disconnect the leads and discard the blotting buffer.
- Open the cassette to reveal the membrane which should show coloured markers, and soak the foam pads and assembly in distilled water.
- Submerge the membrane in 1x TBS, place on an ambient temperature rocking platform for 10 minutes. Repeat these washes three times.
- Prepare 20mL 5% dried skimmed milk solution, per blot, adding 1g of dried skimmed milk powder to 20mL 1x TBS. Mix thoroughly. Pour the 20mL over the blot, which will block the nonspecific binding sites and place on a rocking platform for 30 minutes.
- Prepare 20mL 5% milk solution with primary antibodies, concentrations as recommended by the material safety data sheet, in practise beta-actin 1/5000, SNAP-25 1/1000. Each blot can be treated with different antibodies, as long as the membrane is cut at weight markers specific to the protein, i.e. a blot can be cut at the 37kD marker, the top treated with beta-actin at 45kd and the bottom treated with SNAP-25 antibody at 25kD. Do not combine antibody solutions on one blot, they must be separate.
- Pour the antibody solution over the blot, ensuring it is all covered, cover the container and put on a rocking platform at 4˚C overnight.

Secondary Antibody Incubation

- After overnight incubation in the primary antibody, remove the blot from the container, discard the solution and wash three times for 10 minutes in 1 x TBS, on an ambient temperature rocking platform.
- Prepare 20mL 5% milk solution for each blot, and add 1µl of secondary antibody to the 20mL; 1/20000.
- Pour the solution onto the blot and rotate on a platform for one hour at ambient temperature.

Appendix G ECL Revelation Protocol

Prepare Developer and Fixative

- 18g Fixative in 100mL water
- 16g Developer in 100mL water

Prepare ECL

- Mix equal measures of each solution- 3mL for one membrane. (6mL total)

Take equipment to the dark room

- Saran Wrap (cling film)
- Scissors
- Photographic Film
- Fixative and Developer and ECL Reagents
- Dark Cassette
- Containers

COMPLETE ALL FOLLOWING STEPS IN DARKNESS

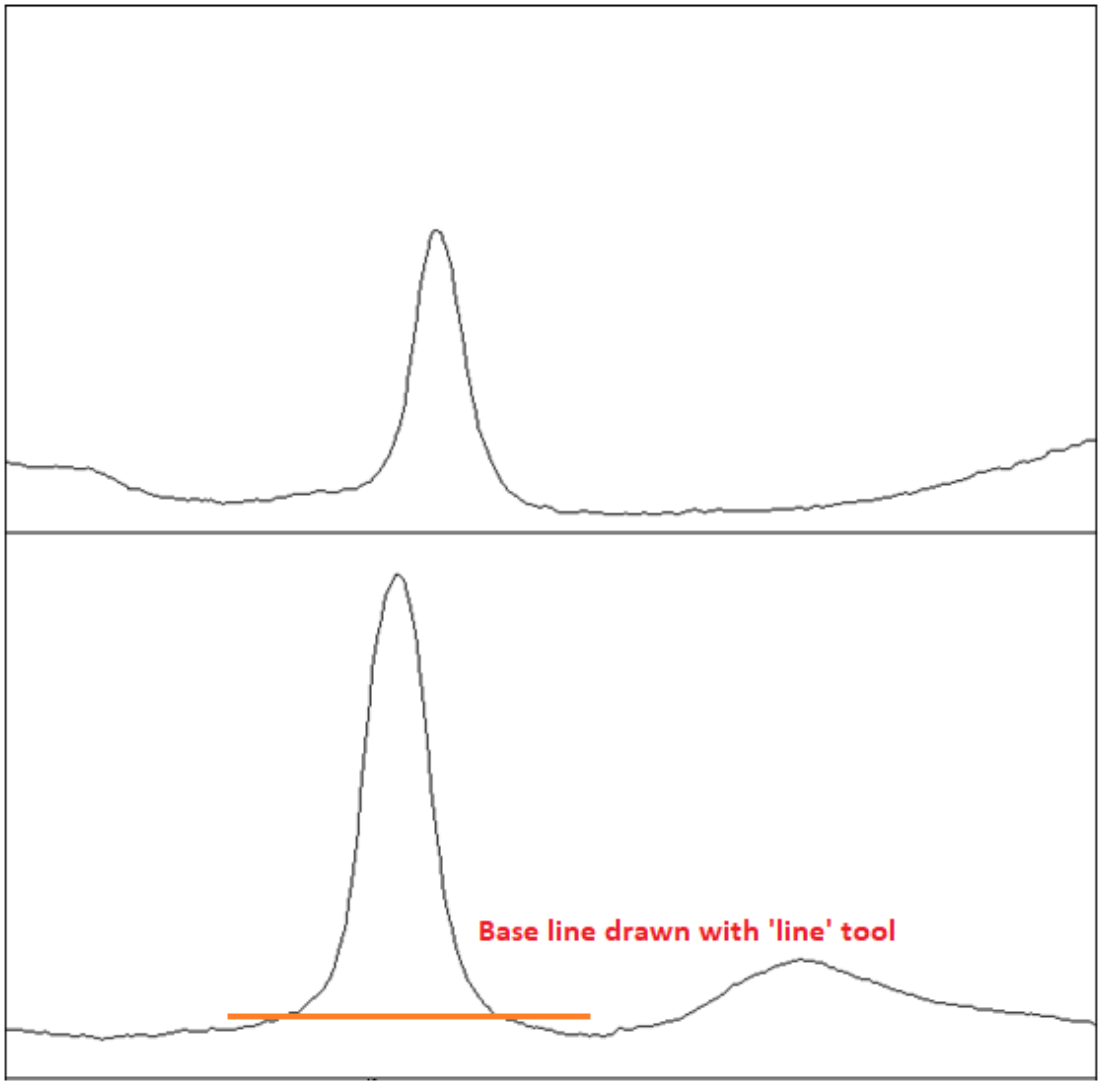
1. Pour the ECL onto the membrane and incubate for **exactly 1 minute**.
2. Drain the ECL from the membrane onto tissue without letting it dry out completely.
3. Wrap the membrane in cling film and place in the cassette (protein side up).
4. Cut a piece of photographic film the same size as the membrane and place on top inside the cassette. Ensure that the film is lined up with the edges of the membrane, placing it correctly first time to minimise moving the blot and risking false results (bands can expose quickly and moving the blot can misplace the band compared to the molecular weight marker, misleading interpretation of protein bands.) **Do not expose the film to light**, and ensure that all excess film is packed away before turning on the light.
5. Expose for required time; Some proteins will require 30 second exposures, up to 10 minutes. For SNAP-25 2-minute exposure is optimal. Beta Actin is optimal at 30 seconds.
6. Move the film to the developer container and submerge entirely. Leave to develop, ensuring it is not overexposed.
7. Rinse the film in water then move to the fixative. This is when the film is able to be exposed to light, the fixative will turn the film transparent and no other bands will develop.
8. Hang the film to dry.

Appendix H Quantification with Image J

1. Download NIH ImageJ software programme.
2. Scan blot images to the computer and copy and paste to Paint programme.
3. Save the image as a 'tif' file to allow document to open in ImageJ.
4. Open the image in the programme, and select IMAGE, TYPE, 8-BIT GREY-SCALE.
5. Select the rectangle tool from the tool bar and draw around the band in the first lane.
6. Ensure that the rectangle is big enough to incorporate all of the bands on the blot image as the size cannot be adjusted for each band.
7. Press ctrl 1, or select ANALYSE, GELS, SELECT 1ST LANE.
8. Drag the rectangle to the next band and press ctrl 2, or select ANALYSE, GELS, SELECT NEXT LANE.
9. Repeat step 8 for each of the bands, pressing ctrl 2 after each one until the final lane has been selected.
10. When the rectangle is on the last lane, press ctrl 3 or select ANALYSE, GELS, PLOT LANES.
11. This generates peaks for each of the bands in a separate pop-up window as shown in the figure below.
12. Select the line tool and draw a line at the base of each of the peaks, trying to exclude any background peaks that may be shown.
13. Repeat this step for all of the bands and then select the 'wand' tool.
14. Click on the inside area of each of the peaks which will make the surrounding line (indicating the calculated area) turn yellow. It should be obvious if an error with the base line is present as the whole area will be encompassed in a yellow line. In this case, repeat step 12 to ensure a proper cut-off point for calculation.
15. Using the wand tool should generate a figure for each of the peaks, in a separate pop-up window, showing the area calculations of each peak, which can be used to show the amount of protein expressed and analysed.

List of References

Peaks generated by ImageJ analysis software; used to quantify intensity of each protein band.



Appendix I Suppliers and Product Codes

Reagent	Supplier	Product Code
L-Alanine/L-Glutamine	Mediatech	25-015-CI
B27	Life Technologies	17504-044
Cryogenic vials	Sigma	V3385-500EA
Donkey Serum	Sigma	D9663
DMSO	Sigma	D2650-5X10ML
EDTA	Sigma	1233508
Fetal Bovine Serum, qualified, heat inactivated	Life Technologies	10500-064
Geltrex	Life Technologies	a1413202-5mL
L-Glutamine	Life Technologies	25030024
Glycerol	Sigma	G2025-100mL
Ham's F-12 Nutrient Mix	Life Technologies	11765-054
LDS sample buffer	Life Technologies	NP0008
MEM EBSS	Life Technologies	11140-035
Methanol	Fisher Scientific	67561
NaCl	Sigma	57653
Neuro-Sure NSC medium	Applied Stem Cell	ASM-4011
Non Essential Amino-Acids	Mediatech	25-025-CI
NP40	Thermo fisher	85124
PBS 1x pH7.4	Life Technologies	10010015

List of References

Reagent	Supplier	Product Code
Pen/Strep	15140	Life Technologies
Poly-D-Lysine	Sigma	P7405
0.01% Poly-Lysine solution	Sigma	P4832
Precision Plus Molecular Marker	Biorad	1610385
Retinoic Acid	Sigma	R2625
RPMI 1640	Life Technologies	21875-034
SDS	Sigma	L3771
SDS/Glycine/TRIS Buffer	Biorad	1610732
Sodium Deoxycholate	Sigma	D6750
TGX Gels 12%	Biorad	4561045
TGX Gels 4-20%	Biorad	4561096
Triethylammonium bicarbonate	Sigma	T7408
TRIS	Sigma	10708976001
Triton X 100	Sigma	X100-100mL
Trypan blue	Sigma	T8154
0.25% trypsin	Life Technologies	15050-065
Vasoactive Intestinal Peptide	Abcam	Ab120180

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